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The Role of Anaerobic Bacteria in Cystic Fibrosis Lung Disease.

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MD Thesis

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Appendix 1: Candidate Thesis Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree MD, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work.

Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed Michelle Murray

RCSI Student Number 11112948

Date 6-4-2014

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Abbreviations

ABA-	anaerobe basal agar
Ab-	antibody
ACE-	acetone
ACT-	α 1 Antichymotrypsin
ADAM-	a disintegrin and metalloproteinases
AAT-	alpha-1 antitrypsin
ASL-	airway surface liquid
ATCC-	American type culture collection
ATF-2 -	activating transcription factor 2
ATP-	adenosine triphosphate
BAL-	bronchoalveolar lavage
BAM-	basal anaerobic medium
BCA-	bacitracin chocolate agar
BSA-	bovine serum albumin
BUT-	butyric acid
C5a-	complement component 5a receptor 1
$CaCL_2{*}2H_2O$-	calcium chloride dihyrate
cAMP-	cyclic adenosine monophosphate
CD 14-	cluster of differentiation 14
CD16-	cluster of differentiation 16
c-DNA-	complementary DNA
CF-BALF-	cystic fibrosis bronchoalveolar lavage fluid

CFBE-	cystic fibrosis bronchial epithelial cells
CpG-	–C–phosphate–G–
CR3-	complement receptor 3
CXC-	chemokine receptor
CXCL-	chemokine ligand 1
CXCR-	chemokine receptor
CFTR-	cystic fibrosis transmembrane conductance regulator protein
DES-	DNase pyrogen free water
DHE-	dihexyl-ether
DMSO-	dimethyl sulphoxide
DNA-	deoxyribonucleic acid
DPBS-	Dulbecco's phosphate buffered saline
E1-	pre exacerbation sample
E2-	post exacerbation sample
EDTA-	ethylenediamine-tetraacetic acid
EGFR-	epidermal growth factor receptor
ELISA-	enzyme linked immunosorbent assay
EP 4-	prostaglandin E receptor 4
ELF-	epithelial lining fluid
ER-	endoplasmic reticulum
ETT-	endotracheal tube
FASL-	Fas ligand
FCS-	foetal calf serum

Fcγ receptors-	FC gamma receptors
FFAR-	free fatty acid receptor
FID-	flame ionisation detector
FITC labelled-	fluorescein isothiocyanate labelled
GADPH-	glyceraldehyde 3-phosphate dehydrogenase
GAGS-	glycosaminoglycans
GC-	gas chromatography
GLP-1-	glucagon-like peptide 1
GPR-	G coupled protein receptors
HA -	hemagglutination assay
HBE-	human bronchial epithelial cells
hCAP 18-	human cathelicidin antimicrobial peptide
HCL-	hydrochloric acid
HDAC-	histone deacetalyse
HRP-	horseradish peroxidase
IBUT-	isobutyric acid
iC3b-	inactivated C3b
ICAM-1-	intercellular adhesion molecule 1
IFN-	interferon gamma
IKK-	IkB kinase
IL-10-	interleukin 10
IL-1β-	interleukin 1 bea
IL-8-	interleukin 8
InpA-	Interpain A

IRAK-	Interleukin-1 receptor associated kinase
IRFD1-	Interferon-related developmental regulator 1
IVAL-	isovaleric acid
JAK1-	Janus kinase 1
K_2HPO_4 -	dipotassium phosphate
KH_2PO_4-	potassium dihydrogen phosphate
KVLB-	kanamycin-vancomycin laked blood agar
LB broth-	lysogeny broth/ Luria-Bertani medium
LL-37-	human cathelicidin
LMA-	laryngeal mask airway
LPS-	lipopolysaccharide
LSC-	laser scanning cytometry
LTB4-	leucotriene B4
m RNA-	messenger RNA
MAPK-	mitogen-activated protein kinases
MEKK1-	mitogen-activated protein kinase kinase kinase 1
MEM-	minimum essential media
$MgSO_4*7H_2O$-	magnesium sulphate heptahydrate
MMP-	matrix metalloproteinase
MNEI-	monocyte/neutrophil elastase inhibitor
MPO-	myeloperoxidase
MRSA-	methicillin resistant *Staphylococcus aureus*
MSSA-	methicillin sensitive *Staphylococcus aureus*
MyD88-	myeloid differentiation primary response gene 88

NaCL-	sodium chloride
NADPH-	nicotinamide adenine dinucleotide phosphate
$NaHCO_3$-	sodium bicarbonate
NAOH-	sodium hydroxide
NE-	neutrophil elastase
NET-	neutrophil extracellular traps
NFAT-	nuclear factor of activated T- cells
NFkB-	nuclear factor kappa-light-chain-enhancer of activated B cells
NIK-	NFkB inducing kinase
NO -	nitric oxide
OLCHC-	Our Ladys Children's' Hospital, Crumlin
PA-	*Pseudomonas aeruginosa*
PAR-2-	protease activated receptor-2.
PBMC-	peripheral blood mononuclear cell
PBS-	phosphate buffered saline
PBST-	phosphate buffered saline-Teen 20
PCR-	polymerase chain reaction
PET -	polyvinylpirrolidone, ethylene glycol tetraacetic acid, trypsin- EDTA solution
PGE_2-	prostaglandin E2
PGP-	peptide proline-glycine-proline
PI3K-	phosphoinositide 3-kinase
PLC-	phospholipase C

PMSF-	phenylmethanesulfonyflonade
PPY-	peptide YY
PRO -	proprionic acid
PRR-	pattern recognition receptors
QUB-	Queen's University, Belfast
RFLP-	restriction fragment length polymorphism
RIPA buffer-	radio immunoprecipitation assay buffer
RNA-	ribonucleic acid
ROS-	reactive oxygen species
RT-PCR-	reverse transcription polymerase chain reaction
RT-	room temperature
S1-	stable, visit 1
S2-	stable visit 2
SCFA -	short chain fatty acids
SDS buffer-	sodium dodecyl sulphate buffer
SDS-PAGE-	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM-	standard error of mean
SEWS-M-	salt ethanol wash solution DNase free
SFM-	serum free media
SLPI-	secretory leukoprotease inhibitor
SMG-	*Streptococcus milleri* group
STAT-	Signal Transducers and Activators of Transcription
TACE-	tumour necrosis factor alpha converting enzyme

TCA-	tricarboxylic acid
Th-1-	T helper cell type 1
TIMPS-	tissue inhibitors of metalloproteases
TLR-	toll like receptor
TNF-α-	tissue necrosis factor alpha
TOPO-	tri-n-octlyphosphine oxide
TRAF-6-	TNF receptor associated factor-6
t-RFLP-	terminal restriction fragment length
UNC-	University of North Carolina
v/v-	volume concentration
VAL -	valeric acid
w/v-	mass concentration

Abstract

Recurrent bacterial infections in Cystic Fibrosis (CF) are the primary cause for morbidity and mortality in CF. Advancements in second generation sequencing and evolution of the lung microbiome has prompted greater interest in other bacteria present in the lung. Anaerobic bacteria have been one of the most common bacteria found on molecular sequencing, their cause and role is as of yet unknown. In our project, we recruited 450 patients prospectively and followed them at both stable and exacerbation timepoints. Their sputum and BAL was cultured and sequenced from both adults and children. The most prevalent bacteria found, *Prevotella melaninogenica* was found to produce proteases that inhibited naturally occurring defensins and anti-microbial peptides and the short chain acids produced by these bacteria were shown to have a pro-inflammatory effect. This provides evidence that certain anaerobic bacteria are pathogenic and these bacteria should be more directly targeted against during an exacerbation.

Presentation and Publications

Poster Presentations:

ATS 2013- Anaerobic bacterial proteases can cleave innate antiproteases and antimicrobials in the lung.

NACFC 2012- Short chain fatty acids which are by products of anaerobic bacteria are involved in CF airway inflammation.

ERS 2012- Proteases from anaerobic bacteria cleave naturally occurring innate antiproteases in CF.

Oral Presentations:

National CF meeting 2013- Role of anaerobic bacteria in CF.

ITS 2012- Effect of anaerobic bacteria in innate immunity in CF lung.

ECFS 2012- Role of short chain fatty acids produced by anaerobic bacteria in CF.

Awards:

ITS Fellowship 2011 Award- Anaerobic bacteria in CF.

Publications:

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Introduction

Chapter 1

1.1 Introduction: Background to Cystic Fibrosis

Although Cystic Fibrosis (CF) affects multiple organ systems, the pulmonary manifestations account for the major morbidity and mortality associated with this condition (Rowe, Miller et al. 2005). Pathogenesis centres upon dysfunction of the cystic fibrosis transmembrane conductance regulator protein (CFTR), a cAMP-activated ATP-binding cassette transporter protein. Expressed in submucosal glands and the apical membranes of epithelial cells, CFTR normally functions as a chloride ion channel (Sheppard and Welsh 1999).

Over 1800 CFTR mutations have been identified to date and a subgroup of these are disease-causing mutations and can be grouped into six classes (I-VI) depending on whether the mutations affect CFTR expression, processing, activity or a combination of these (Lommatzsch and Aris 2009). Two-disease causing mutations are required to cause disease. The ΔF508 mutation accounts for ~70% of CFTR alleles worldwide. Correlations between CFTR genotype and disease manifestations and progression are weak highlighting that other factors are important. Indeed genetic modifiers have been reported to play a substantial role in determining FEV_1. The most promising candidate modifier genes for CF include interleukin-8 (Hillian, Londono et al. 2008), CXCR1/2 (Kormann, Hector et al. 2011), mannose binding lectin (Garred, Pressler et al. 1999, Dorfman, Sandford et al. 2008), transforming growth factor beta 1 (Drumm, Konstan et al. 2005, Dorfman, Sandford et al. 2008), interferon related developmental regulator 1 (Potempa, Potempa et al. 2009) and endothelin receptor type A (Cutting 2010,

Darrah, McKone et al. 2010), whilst the SERPINA1 Z allele is a risk factor for liver disease in CF patients (Bartlett, Friedman et al. 2009).

Where CFTR's function is diminished or absent, a cycle of events ensue including sodium hyper-absorption, mucus hypersecretion, impaired mucociliary clearance and pathogenic colonization by amongst others, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenza* and *Aspergillus fumigatus*. This in turn leads to the clinical picture of recurrent infections, persistent chronic neutrophilic inflammation causing bronchiectasis and airway destruction culminating in respiratory failure and premature death (Davis, Drumm et al. 1996).

1.2 CF Dysregulated Defences

1.2.1 The Lung Epithelium

The lung and its alveolar membrane constitute an enormous surface area in direct contact with the outside environment. In addition to its continual exposure to a large number of airborne or microbial molecules, the lung can also be confronted with pathogens via the blood stream and as such, acts as a major portal for the entry of invading pathogenic microorganisms. Despite this, the lung possesses a unique ability to maintain relative sterility due to a number of factors including the pulmonary epithelium and other innate immune cells that make up the pulmonary innate immune defence. The epithelial lining of the lung acts as an effective physical barrier to infection but additionally plays an active role in innate

immune defences against invading pathogens particularly relevant in the CF context. Signalling in the airway epithelium in response to bacterial insults involves the activation of multiple receptors and signalling pathways leading to the eventual release of numerous cytokines and chemokines that serve to recruit phagocytic cells (Gomez and Prince 2008). Pulmonary innate immune mechanisms include airway surface liquid (ASL) and its mucociliary escalator, proteases, anti-proteases, antimicrobials and pattern recognition receptors such as the toll-like receptors (TLRs).

Intact bacteria are rarely in direct contact with the airway epithelia owing to protection afforded by mucin and subsequent removal by the mucociliary escalator however in pathological states like CF, components of the bacterial cell wall or virulence factor such as flagella are major activators of pro-inflammatory signals for example through TLRs due to inadequate clearing of the mucosal layer.

Mucus is made up of a variety of components including water, ions, proteins, lipids and polymeric glycoproteins termed 'mucins'. Nineteen human mucin genes have been described to date with the most important airway mucins the secreted Muc5AC and Muc5B produced by goblet cells of the superficial airway epithelium. Expression is augmented in the CF lung and the overall composition of CF mucus is altered as a result due to an increased content of macromolecules such as DNA, filamentous actin, lipids, and proteoglycans. Together these contribute to mucus plugging and pathogenesis of infection within the CF lung by facilitating *Pseudomonas aeruginosa, Staphylococcus aureus,*

Burkholderia cepacia, *Prevotella* species, *Candida* species, and *Aspergillus* species colonisation amongst others (Rose and Voynow 2006, Voynow and Rubin 2009).

Mutations in CFTR lead to decreased bacterial clearance, intrinsic hyperinflammation and reduced bacterial killing (Chmiel and Davis 2003). Whether CFTR dysfunction results directly in an increased predisposition to infection or whether inflammation arises independent of infection remains to be established. What is clear, is that abnormal ion conductance within the airway consequently results in a decreased airway surface liquid (ASL) height leading to luminal dehydration and increased mucus presence and viscosity, which in turn facilitates chronic microbial colonisation attracting large numbers of activated neutrophils into the airway. The lack of effective response to chronic infection and colonization in CF is explained at least in part by the lack of functional cellular CFTR, as bacterial clearance has been reported to require CFTR modulated IL-1β release (Reiniger, Lee et al. 2007). Additionally, elevated endogenous furin levels in CF-BALF illustrate a cellular susceptibility to *Pseudomonas* exotoxin cytotoxicity (Ornatowski, Poschet et al. 2007). A further disruption to the existing protease-antiprotease balance in CF consequently occurs due to excessive quantities of both neutrophil- and bacterial-derived proteases within the CF lung that interferes with the normal innate immune response (Greene and McElvaney 2009).

Airway epithelial cells in addition to their role in regulating lung homeostasis, sense pathogens by their transmembrane and cytosolic pattern

recognition receptors (PRRs). A major PRR in the lung, is the toll-like receptor family whose mucosal expression allows activation upon superficial contact with microbial derived factors such as lipopolysaccharide (LPS) or flagellin from *Pseudomonas aeruginosa* (Greene, Carroll et al. 2005). TLRs are known to be both present and functional within the CF airway (Greene, Carroll et al. 2005). The predominant TLR expressed on bronchial and/or tracheal epithelia is TLR2 with other TLRs namely TLR3, TLR4 and TLR5 mobilised to the cell surface upon activation illustrating that subcellular expression exists and is cell type dependent. For instance, TLR2 is expressed apically whilst TLR4 and TLR5 exist on the basolateral surface (Greene, Carroll et al. 2005). In the bronchial epithelium however, TLR4 takes up an intracellular position. In addition to the TLRs, other non-TLR transmembrane receptors on airway epithelial cells such as the complement receptors and the cytosolic NOD receptors may have adjunct roles in microbial recognition and the overall pulmonary innate response (Figure 1.1).

1.2.2 Protease-anti-protease defences in CF inflammation

Proteases within the healthy lung fulfil basic homeostatic roles and regulate key cellular functions. The principal classes of protease present in the lung are the serine, cysteinyl, aspartyl and metalloproteases. In the past, proteases were viewed as causes of lung tissue destruction but it is now apparent that they regulate processes as diverse as tissue remodelling, mucin expression, neutrophil chemotaxis, bacterial killing and effect interaction with innate immune receptors.

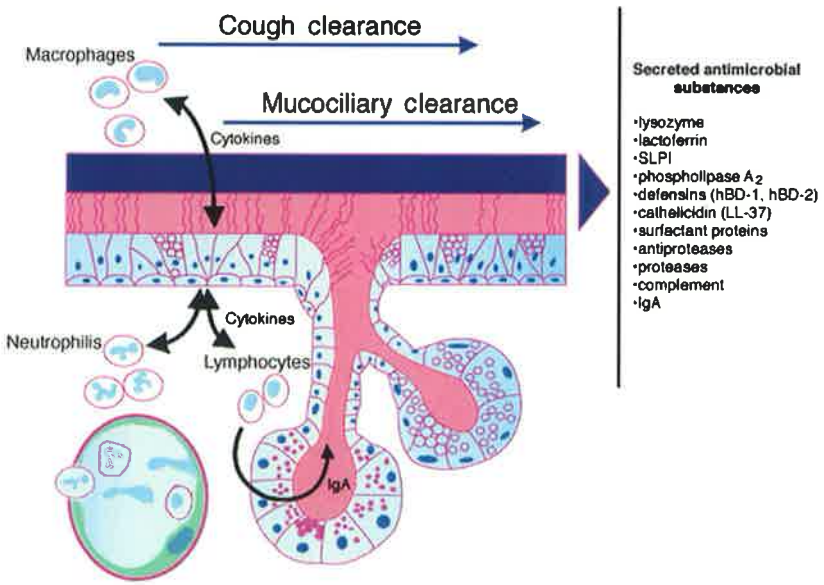


Figure 1.1: Host Defence Mechanisms of Respiratory Epithelium
Cough and cilia mechanically remove inhaled debris and microorganisms entrapped in mucus, the process known as mucociliary clearance. Multiple agents with pro- and anti-inflammatory, as well as antimicrobial, activities are secreted by epithelial cells and function as an innate immune barrier. Macrophages, B and T cells, neutrophils, and also epithelial cells represent cellular components of the innate and adaptive immune systems (Adapted from Bals et al, Journal of Clinical Investigation, 2002).

1.2.2 The CF neutrophil

From childhood, CF is characterised by a persistent and sustained neutrophilic influx however such cells fail to adequately clear microbes from the lung, hence permitting colonisation, recurrent infection, airway destruction, bronchiectasis and respiratory failure (McElvaney, Nakamura et al. 1992, Birrer, McElvaney et al. 1994). Neutrophil dysfunction in CF is associated with cellular necrosis and subsequent protease release leading to a subjugation of the anti-protease defence systems (McElvaney, Hubbard et al. 1991). Whether such dysfunction in the CF neutrophil can be attributed to a primary cell defect or secondary upon exposure to infection and inflammation in the CF lung, is an area of ongoing research.

The presence of CFTR on the neutrophil membrane is still a matter of controversy. While CFTR mRNA transcripts have been described in neutrophils (Painter, Valentine et al. 2006) to a similar extent as that observed in other cell types such as monocytes, not all work performed in this area has confirmed this (Yoshimura, Nakamura et al. 1991, Morris, Doull et al. 2005). In support of its presence however, immunological approaches have shown the full mature and glycosylated form of the protein in a population of purified neutrophils (Painter, Valentine et al. 2006).

Genetic defects may lead to a dysregulated CF neutrophil activity *in vivo* and the presence of a myeloperoxidase promoter polymorphism (-463A) has been associated with significantly worse clinical CF disease however this was

later found not to be exclusive to CF but in association with other chronic diseases as diverse as vasculitis, heart failure and lung cancer (Reynolds, Sermet-Gaudelus et al. 2006). Genome wide research has also illustrated a modifier role for interferon-related developmental regulator 1 (IFRD1) in CF pathogenesis predominantly through its effects on neutrophil function again supportive of genetic basis for cellular dysfunction (Potempa, Potempa et al. 2009).

Neutrophil chemotaxis is defined by cell movement to sites of tissue injury or infection along a concentration gradient. This process is carried out in a stepwise fashion including adhesion, rolling and migration as major steps in the process (Ley, Laudanna et al. 2007). Abnormalities in some of these steps have been described in the CF context; elevated levels of ICAM-1, E- and P-selectin are detected in CF serum (De Rose, Oliva et al. 1998) when compared to non-CF cohorts and CF neutrophils obtained from those in both stable and exacerbation phases of disease illustrate significantly less L-selectin shedding in comparison to non-CF or inflammatory control subjects (Russell, McRedmond et al. 1998). Additionally, neutrophil migration in CF appears to be mediated by a CD-18 independent mechanism and is driven by elevations in the chemoattractants found within the CF environment including leukotriene B_4 and IL-8 (Bodini, D'Orazio et al. 2005, Mackarel, Plant et al. 2005).

The neutrophil driven respiratory burst contributes to bacterial killing under normal circumstances however the CF neutrophil generates constitutively higher levels of O_2^- following an exacerbation that contributes to inflammatory damage

in the airway (Brockbank, Downey et al. 2005). It has also been noted that a greater level of MPO-dependent activity and chloramine production is detected extracellularly from cells obtained from CF homo- and heterozygotes (Cantin and Woods 1993, Witko-Sarsat, Allen et al. 1996). Defective intraphagolysosomal HOCl production has been reported and may be attributed to the lack of CFTR activity in disease as the ion channel is critical in the processes of neutrophil chlorination following bacterial phagocytosis (Painter, Valentine et al. 2006, Painter, Bonvillain et al. 2008). CF airway neutrophils have been shown to be unresponsive to the anti-inflammatory IL-10 signal while intravascular systemically circulating CF neutrophils are particularly sensitive to priming agents such as *Pseudomonas* alginate, TNF-α and IL-8, all of which cause greater release of MPO and NE as compared to non-CF neutrophils (Pedersen, Kharazmi et al. 1990, Koller, Urbanek et al. 1995, Taggart, Coakley et al. 2000, Petit-Bertron, Tabary et al. 2008). Additionally, unopposed proteolytic activity in the CF airway cleaves CXCR1 on neutrophils and in turn disables the bacterial killing capacity of airway neutrophils (Hartl, Latzin et al. 2007).

Despite adequate CR3 expression, the airway CF neutrophil demonstrates a diminished phagocytic capability as compared to circulating or control neutrophils (Morris, Doull et al. 2005). Whether such defects in the bacterial killing abilities are dependent on the inflammatory state in the CF lung remains to be established however communication between opsonising agents and surface receptors are influenced by the high levels of NE present that cleave CXCR1, Fcγ receptors and ic3b, all important molecules in the phagocytic process (Tosi

and Berger 1988, Berger, Sorensen et al. 1989, Tosi, Zakem et al. 1990, McElvaney, Hubbard et al. 1991, Birrer, McElvaney et al. 1994, Moraes, Plumb et al. 2006, Hartl, Latzin et al. 2007). In support of this latter observation, incubation of non-CF neutrophils with NE reproduces the impaired phagocytic state seen in CF (Alexis, Muhlebach et al. 2006). Furthermore, CF airway neutrophils lose surface expression of CD14 and CD16 (Tirouvanziam, Gernez et al. 2008). In addition to phagocytosis, the degranulation process has also been illustrated as defective in CF, as increased levels of the primary granule components NE and MPO are detectable in the extracellular environment following stimulation of systemic CF neutrophils with CF BALF, IL-8 or TNF-α (Koller, Urbanek et al. 1995, Taggart, Coakley et al. 2000). Moreover, an altered intracellular pH within these neutrophils impacts on mechanisms involved in the degranulation process itself (Coakley, Taggart et al. 2000, Coakley, Taggart et al. 2002).

Intravascular CF neutrophils show decreased apoptosis owing to decreased Fas and FasL (Downey, Brockbank et al. 2007). Further supporting this is the increased coronin-1 observed, however this not exclusive to CF and can be observed in other inflammatory states including sepsis and the systemic inflammatory response syndrome (Keel, Ungethum et al. 1997, Mercer-Jones, Heinzelmann et al. 1997, McKeon, Condliffe et al. 2008, Moriceau, Kantari et al. 2009, Moriceau, Lenoir et al. 2010). Despite this latter observation, neutrophils obtained from CF heterozygotes show delayed apoptosis supportive of a primary defect in the control of cell death during disease and CFTR mutations have been independently linked to a negative impact upon apoptosis (Jungas, Motta et al.

2002, Moriceau, Lenoir et al. 2010). In addition to potentially delayed apoptosis, the clearance of apoptotic cells by macrophages in the CF airway is further hindered by CF associated factors including NE cleavage of the phosphotidyl serine receptor and the presence of pyocyanin, a *Pseudomonas* associated exotoxin (Bianchi, Prince et al. 2008).

Within the CF lung, the inflammatory response to a defined bacterial load is greater compared to the normal lung (Davis, Drumm et al. 1996, Chmiel and Davis 2003). Pulmonary inflammation in CF is characterised by a sustained accumulation of neutrophils; high proteolytic activity and elevated levels of chemokines such as IL-8 (Sagel, Chmiel et al. 2007). The inflammation in this context can be affected by a variety of neutrophil chemotactic factors including ceramide, chemotactic tripeptides (PGP) (Greene 2010), C5a and the leukotriene LTB4 which together contribute to the highly proinflammatory milieu in the CF lung. In addition, the CF lung is a TLR agonist-rich environment, represented by microbial-derived factors and neutrophil elastase (NE) (Greene, Branagan et al. 2008). In CF, our group has shown that NE-induced activation of TLR signalling can additionally be mediated by EGFR ligand generation and EGFR activation (Bergin, Greene et al. 2008). The protease rich environment of the CF lung negatively impacts on antimicrobial protein activity. Defensins, lactoferrin, LL-37 and SLPI are all susceptible to proteolytic degradation particularly by cystinyl cathepsins and to a lesser extent by NE (Bergsson, Reeves et al. 2009).

Due to the high numbers of neutrophils present within the inflamed CF airway, coupled with their inherent ability to release reactive oxidant species

(ROS), the redox balance in the CF setting is altered. Furthermore, a reduction of glutathione levels on the respiratory epithelial surface multiplies the effects of these described environmental influences on inflammation and together leads to oxidative damage (Roum, Buhl et al. 1993). Additional disease based factors such as CFTR genotype and gender coupled with microbial influences such as *Pseudomonas* mucoidy and co-colonisation with *Staphylococcus*, *Burkholderia* or anaerobic bacterial species all contribute to determining the inflammatory state within the CF airway at any given stage of disease.

Peptide proline-glycine-proline (PGP) is a proinflammatory chemotactic peptide regulated by metalloproteases (MMP), MMP 8 and MMP 9 in CF. PGP is a breakdown of extracellular matrix protein collagen. There is evidence now, that fragments of a number of extracellular matrix proteins, including those derived from collagen and elastin, are important in regulating the recruitment of inflammatory cells to the lung (Gaggar, Jackson et al. 2008). PGP (and N-acetylated PGP) has been shown to act as a neutrophil chemoattractant via CXC receptors 1 and 2 on neutrophils (Gaggar, Jackson et al. 2008). PGP in CF sputum, could have benefits as a potential biomarker, as it has been shown to have elevated levels during acute exacerbation and these levels fall with antibiotic treatment (Gaggar, Jackson et al. 2008).

1.2.3 Neutrophil elastase

NE is the major protease released by neutrophils in the CF lung and functionally possesses multifaceted effects that impact upon infection and inflammation in the CF microenvironment (Kelly, Greene et al. 2008). It is

capable of up-regulating the expression of other proteases including metalloproteases and cysteinyl cathepsins (Geraghty, Rogan et al. 2007) in addition to inactivating serine antiproteases (elafin and SLPI) decreasing their anti-inflammatory and immunomodulatory properties (Kelly, Greene et al. 2008, Weldon, McNally et al. 2009). In tandem with proteinase-3, macrophage-derived metalloelastases and elastolytic proteases all expressed by *Pseudomonas aeruginosa* (*P. aeruginosa*), NE can promote secretion of mucus and degrade surfactant proteins and antimicrobials. Additionally, NE can directly injure epithelial cells and reduce ciliary beat frequency, cleave haemoglobin, complement components and immunoglobulins and interfere with effective neutrophil killing of microbes (Kelly, Greene et al. 2008). In the CF lung NE-induced activation of TLR signalling is mediated in conjunction with EGFR activation (Bergin, Greene et al. 2008). Not all NE mediated events are deleterious. NE contributes to bacterial death on distinct two levels, first the intracellular killing of gram-negative bacteria by neutrophils and secondly once released extracellularly, NE comprises a key component of neutrophil extracellular traps (NETs). NETs are involved in host defence (Brinkmann, Reichard et al. 2004) and bind bacteria (gram positive and negative) allowing neutrophil delivery of high concentrations of serine proteases that degrade virulence factors protecting the host and ultimately kill the bacterium.

Certain bacterial virulence factors may counteract NETs for instance the expression of DNAses that degrade the NET-backbone, expression of a capsule which can reduce bacterial trapping and modulation of cell-surface charge

(Buchanan, Simpson et al. 2006, Wartha, Beiter et al. 2007). Neutrophil-derived oxidants and proteases overwhelm the anti-protease defence systems on the respiratory epithelial surface. In the past, this was seen mainly as a pure anti-protease defence but more recently the immunomodulatory and anti-inflammatory properties of these anti-proteases suggest that their degradation by proteases has other more profound effects (Kelly, Greene et al. 2008, Weldon, McNally et al. 2009).

Other serine proteases include proteinase 3 and cathepsin G which are also released by activated neutrophils into the chronic inflammatory milieu in CF albeit in less abundance as compared to NE (Goldstein and Doring 1986, Witko-Sarsat, Halbwachs-Mecarelli et al. 1999). In addition to inducing the transcription of specific proteases, NE and these other serine proteases activate MMPs. For example NE, proteinase 3 and cathepsin G can activate the latent 72 kDa MMP-2 zymogen via membrane type I MMP (Imai, Yokohama et al. 1995, Ferry, Lonchampt et al. 1997, Shamamian, Schwartz et al. 2001). NE can also activate proMMP-7, MMP-9 and procathepsin B and members of the ADAM (a disintegrin metalloprotease) and meprin families (Dalet-Fumeron, Guinec et al. 1993, Imai, Yokohama et al. 1995, Ferry, Lonchampt et al. 1997, Kohri, Ueki et al. 2002, Bergin, Greene et al. 2008). The interaction between proteases and NE is described below (Figure 1.2).

In tandem with other pulmonary proteases NE generates bioactive molecules from collagen and in a collaborative effort with elastolytic proteases including proteinase-3, macrophage-derived metalloelastases and pseudomonal

proteases, it promotes mucus secretion, degrades antimicrobials and surfactant proteins all of which play a role in the normal function of innate pulmonary defence (Nakamura, Yoshimura et al. 1992, Walsh, Greene et al. 2001, Kohri, Ueki et al. 2002, Devaney, Greene et al. 2003, Shao and Nadel 2005, Shao and Nadel 2005, Bergin, Greene et al. 2008).

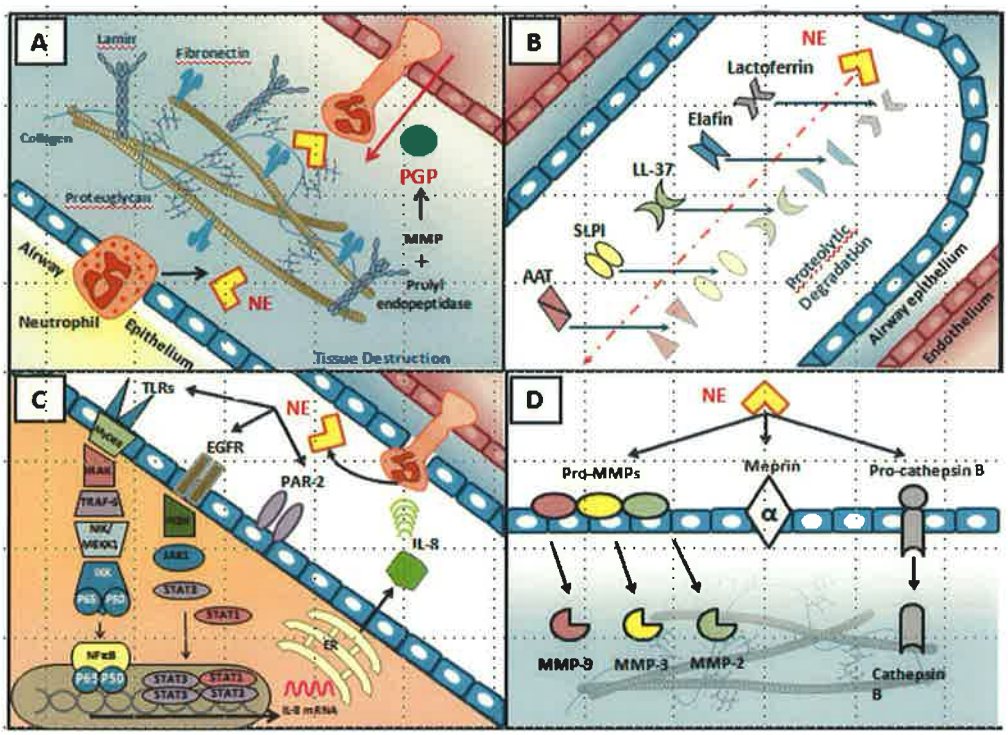


Figure 1.2: Schematic representation of the effects of neutrophil elastase (NE) in the CF lung.

(A) NE mediates destruction of the lung extracellular matrix and production of the chemoattractant PGP (proline-glycine-proline) increases neutrophilic influx to the airway. (B) NE cleaves important anti-proteases and anti-microbial proteins in the CF lung including alpha-1 antitrypsin (AAT), secretory leukoprotease inhibitor (SLPI), the cathelicidin: LL-37, elafin and lactoferrin (C) NE activates TLR (Toll-like receptor) signalling in conjunction with EGFR (Epidermal growth factor receptor) as well as PAR-2 (Protease activated receptor-2). This leads to up-regulation of pro-inflammatory cytokines such as interleukin 8 (IL-8). (D) NE induced activation of matrix metalloproteases (MMPs), meprin-α and cathepsin B. MyD88: Myeloid differentiation primary response gene (88); IRAK: Interleukin-1 receptor associated kinase; TRAF-6: TNF receptor associated factor-6; NIK: NFkB inducing kinase; MEKK1: Mitogen-activated protein kinase kinase kinase 1, IKK: IkB kinase; NFkB: PI3K: Phosphoinositide 3-kinase; JAK1: Janus kinase 1; STAT: Signal Transducers and Activators of Transcription; ER: Endoplasmic reticulum. (Adapted from book in press, Chapter: Interactions between infection and inflammation in CF airway. Chotirmall SH, Murray MA et al.Cystic fibrosis textbook. Hodson Margaret; Bush Andrew.)

1.3 Antiproteases

Pulmonary protease activity is counter regulated by antiproteases whose function is compromised in the CF setting. There are three major serine antiproteases in the lung - alpha-1 antitrypsin (AAT), secretory leucoprotease inhibitor (SLPI) and elafin. Functionally, all have broad capabilities because in addition to their anti-protease function they possess immunomodulatory, anti-inflammatory and antimicrobial properties. Monocyte /neutrophil elastase inhibitor (MNEI) is yet another pulmonary serine protease inhibitor with activity against NE, cathepsin G and proteinase 3 (Cooley, Takayama et al. 2001). The cysteinyl cathepsins are inhibited by the endogenous cystatin families that exist intracellularly, extracellularly and intravascularly (Rawlings, Tolle et al. 2004) whilst the tissue inhibitors of metalloproteases (TIMPS) regulate the activities of MMPs and also have an inhibitory role against several members of the ADAM family (Huovila, Turner et al. 2005).

1.3.1 A1AT

A1AT is an acute phase 52kDa 418 amino acid glycoprotein whose primary role is as an NE inhibitor (Travis, Owen et al. 1985). It is primarily synthesised by hepatocytes (Rogers, Kalsheker et al. 1983). Additionally, it is released in smaller quantities by immune cells (Molmenti, Perlmutter et al. 1993) and within the pulmonary microenvironment by bronchial epithelial cells (Mason, Cramer et al. 1991, Venembre, Boutten et al. 1994, Cichy, Potempa et al. 1997, Hu and Perlmutter 2002, Mulgrew, Taggart et al. 2004). A1ATs possesses an impressive broad range of antiprotease function in addition to its role as the

principal serine protease inhibitor including inhibition of trypsin, chymotrypsin, cathepsin G, plasmin, thrombin, tissue kallikrein, factor Xa, plasminogen, proteinase 3 and TACE (Bergin, Reeves et al. 2010). It's abilities also include decreasing TNFα and MMP signalling in alveolar macrophages (Churg, Wang et al. 2003), an impairment of LPS-induced monocyte activation and the restriction toward apoptosis (Daemen, Heemskerk et al. 2000, Ikebe, Akaike et al. 2000, Ikari, Mulvihill et al. 2001). In addition it has an immunomodulatory role, where it inhibits neutrophil superoxide production.

AAT is totally inactivated in the CF lung, being complexed with NE, cleaved by proteases or oxidatively inactivated by oxidants. It is unsurprising therefore that AAT augmentation by aerosolisation is considered an excellent therapeutic target in CF. Aerosolisation of AAT to CF patients suppresses NE in epithelial lining fluid (ELF) and restores its anti-NE capabilities suggestive that it may augment host defences in the CF context (McElvaney, Hubbard et al. 1991). It is however crucial to note that even this exogenous A1AT is susceptible to both cleavage and oxidative inactivation. Following oxidation, its anti-NE capacity becomes negligible whilst both cathepsin L and *Pseudomonas* elastase are known to cleave AAT (Morihara, Tsuzuki et al. 1984, Johnson, Barrett et al. 1986) both factors of importance in the CF airway. It is for these latter reasons that it is yet to be found in routine use in the management of protease induced inflammation in CF.

1.3.2 SLPI

SLPI is a cationic, non-glycosylated serine proteinase inhibitor that inhibits NE, cathepsin G, trypsin, chymotrypsin, chymase and tryptase (Mc Elvaney and Crystal 1997 , Doumas, Kolokotronis et al. 2005). Constitutively expressed at many mucosal surfaces, it is produced by numerous different cell types and in particular the respiratory epithelium. In addition to its antiprotease activity, SLPI has potent antimicrobial and antiviral properties. Due to its high cationicity, SLPI can disrupt the microbial cell membranes of *S. aureus* (*Staphylococcus aureus)*, *P. aeruginosa* and *C.albicans (Candida albicans)* all important microorganisms in CF (Wiedow, Harder et al. 1998, Williams, Brown et al. 2006).

From an immunomodulatory perspective, SLPI decreases production of prostaglandins and MMP-1 and -9 in monocytes (Zhang, DeWitt et al. 1997), inhibits interferon-γ-induced cathepsin S expression (Geraghty, Greene et al. 2007) and blocks activity of bacterial LPS with the use of cytoplasmic and nuclear mechanistic approaches (Jin, Nathan et al. 1997, Ding, Thieblemont et al. 1999). Whilst the extracellular role of SLPI is recognised, intracellular roles have been reported and are relevant in the CF context (McNeely, Shugars et al. 1997, Taggart, Cryan et al. 2005). SLPI can be internalised by monocytes and influence LPS induced responses (Taggart, Greene et al. 2002, Mulgrew, Taggart et al. 2004) by preventing proteolytic degradation of IRAK-1, IκBβ and IκBα and as a direct result of SLPI binding to NFκB p65-subunit binding sites on DNA it permits the restriction of transcription of NFκB-regulated genes (Taggart, Greene et al. 2002, Taggart, Cryan et al. 2005). Its lack in CF sputum has been touted for a

potential role as a biomarker as it has been described to be both cleaved and inactivated by NE and to a lesser extent cathepsins present in *P. aeruginosa* positive CF lung secretions (Weldon, McNally et al. 2009, Sagel, Wagner et al. 2012). From a therapeutic perspective, SLPI offers a viable target in the CF context however challenges with its delivery are similar to those encountered with A1AT and continue to be actively researched (Zani, Tanga et al. 2011).

1.3.3 Elafin

Elafin, known as SKALP (skin-derived antileukoprotease) or ESI (elastase-specific inhibitor) shares homology with SLPI. Principally secreted by epithelial surfaces including the lung (Alkemade, Molhuizen et al. 1994, Nonomura, Yamanishi et al. 1994, Sallenave, Shulmann et al. 1994, Pfundt, van Ruissen et al. 1996, van Wetering, van der Linden et al. 2000), it possesses the ability to protect tissues from enzymatic degradation by inhibiting proteinase 3 and human granulocytic elastase but not trypsin, chymotrypsin, or granulocytic cathepsin G differentiating it from SLPI (Hochstrasser, Albrecht et al. 1981, Wiedow, Luademann et al. 1991). Expression can be induced in response to proinflammatory stimuli such as LPS or by TNF-α and IL-1β (Sallenave, Shulmann et al. 1994) (Reid, Marsden et al. 1999, Simpson, Cunningham et al. 2001). Like other anti-proteases described, it has anti-inflammatory and anti-bacterial activities particularly against the main CF pathogens *P. aeruginosa* and *S. aureus* (Simpson, Maxwell et al. 1999). Additionally, a dose dependent reduction in LPS-induced neutrophil influx can occur where elafin is abundant whilst it can independently inhibit MMP-9 production and prevent CXCL1 and

CXCL2 release (Simpson, Maxwell et al. 1999). Importantly in a high NE environment such as the CF lung, it undergoes cleavage. Although such cleavage has no effect on its anti-NE capacity it does compromise its immobilisation and ability to bind LPS (Guyot, Butler et al. 2008). Such features have implications *in vivo* and consequently in the infective and inflammatory CF context.

The pulmonary anti-proteases SLPI and elafin also possess anti-TLR properties. SLPI can inhibit TLR-induced cytokine release by impairing TLR2 and TLR4 responses in monocytes (Mulgrew, Taggart et al. 2004). Furthermore, the existence of a novel endogenous negative feedback loop is evidenced by the fact that LPS is known to up-regulate SLPI production in macrophages (Jin, Nathan et al. 1998). The underlying mechanisms by which SLPI can interfere with LPS signalling involve an impairment of degradation of IκBα without affecting phosphorylation or ubiquitination or alternatively by competitive inhibition of p65 NFκB sites on DNA (Taggart, Greene et al. 2002, Taggart, Cryan et al. 2005). Trappin-2 (pre-elafin) following proteolytic cleavage releases mature elafin that can act in an anti-inflammatory fashion in monocytes via impairment of AP-1 and NFκB activation by effects on the ubiquitin-proteasome pathway (Guyot, Zani et al. 2005, Butler, Robertson et al. 2006).

1.3.4 Anti-Microbial Peptides

Antimicrobial peptides including the defensins, cathelicidins, SLPI, lactoferrin and lysozyme are critical effector molecules at the interface between infection and CF lung's innate immune defence system. In addition to direct

bacterial killing activity some of these have anti-biofilm, anti-inflammatory and anti-viral properties (Rogan, Geraghty et al. 2006).

1.3.5 Defensins

Defensins are low molecular weight non-glycosylated cationic proteins (Ganz 2003). The α-defensins are expressed by neutrophils while epithelial cells express β defensins (HBDs). Differentiated by structure, functionally α- and β-defensins both have overlapping yet distinctive activities. HBDs possess broad antimicrobial functions against bacteria, viruses and fungi owing to their ability to disrupt microbial membranes rich in negatively charged phospholipids. While their main effect are 'salt-sensitive' functionally they possess the capability to influence immune cell chemotaxis, mast cell activation and cell cytotoxicity. HBDs have been extensively studied in the context of CF. An increased salt concentration in ASL secretions decreases β-defensin activity (Smith, Travis et al. 1996, Goldman, Anderson et al. 1997) however whether this operates *in vivo* has been questioned as a salt independent impairment of its antimicrobial activity is clearly demonstrated (Bals, Weiner et al. 2001). Compromised defensin capabilities in CF depends upon its fatal interaction with mucins and DNA released from necrotic cells in addition to protease degradation specifically by cystinyl cathepsins, all components of the chronic inflammatory environment typical of the CF lung (Taggart, Greene et al. 2003).

1.3.6 Cathelicidins

Cathelicidins are recognised by their conserved N-terminal portion whilst the C-terminal anti-microbial moiety varies in structural terms. Known as

multifunctional peptides, they exist as inactive propeptides, with activation occurring upon release of tissue specific processing enzymes that act on their C-terminal component. The only human and relevant cathelicidin in the CF context is hCAP-18/LL-37. Interactions between LL-37 and the mucin content of the ASL may increase infection rates in CF (Smith, Travis et al. 1996, Felgentreff, Beisswenger et al. 2006) whilst levels quantified in CF bronchoalveolar lavage (BAL) correlate with disease severity (Chen, Schaller-Bals et al. 2004). The hormonally active form of Vitamin D is known to upregulate anti-microbial peptide expression particularly cathelicidin. Hence, vitamin D deficiency in CF contributes to persistent infection thought to be due to the lack of cathelicidin peptides (Yim, Dhawan et al. 2007). Furthermore, LL-37's fatal interactions with DNA, filamentous F-actin and lipopolysaccharide (LPS) all additionally significantly compromise its anti-microbial activity in CF sputum hence methods to restore its functional capability such as nebulised hypertonic saline administration continue to be investigated (Weiner, Bucki et al. 2003, Bucki, Byfield et al. 2007).

Glycosaminoglycans (GAGs) play a crucial role in regulating ECM structure, function, and inflammatory response. However, there is increasing evidence of dysregulated GAG expression and sulphation in the pathophysiology of CF lung disease in CF BALF and their impact on antimicrobial activity. LL-37 is bound to glycosaminoglycans which renders it inactive. Hypertonic saline by forming bonds between LL37 and GAGs has been demonstrated to release LL-37 from its bound form making it active again (Bergsson, Reeves et al. 2009, Reeves, Bergin et al. 2011).The role of GAGs in other aspects of CF

inflammation needs to be evaluated. IL-8, for example, unlike LL-37, which is found to be extensively bound to GAGs in the CF lung, remains active as a neutrophil chemoattractant. Hypertonic saline again disrupts the bond releasing IL-8 for protein degradation and this paves the way for inflammation resolution (Reeves, Williamson et al. 2011).

1.3.7 Lactoferrin

The iron chelator, lactoferrin is stored and subsequently released from secondary granules of the neutrophil at sites of infection and its associated inflammation. From a CF perspective, its bactericidal and bacteriostatic roles extend to the inhibition of *P. aeruginosa* biofilms (Singh, Parsek et al. 2002). One of the explanations for its anti-microbial effects is its affinity for elemental iron, an essential growth factor for microorganisms including *P. aeruginosa*. A second mechanism is lactoferrin's ability to bind to bacterial membranes and altering their permeability allowing injury and death (Travis, Conway et al. 1999). In addition to effects against viruses and fungi, (Soukka, Tenovuo et al. 1992, Harmsen, Swart et al. 1995, Beljaars, van der Strate et al. 2004, Shin, Wakabayashi et al. 2005) it possesses immunomodulatory properties (Baveye, Elass et al. 1999). As a potent anti-inflammatory (Conneely 2001), it decreases expression of cell adhesion molecules such as ICAM-1 and E-selectin, (Baveye, Elass et al. 2000) binds TLR9 agonists unmethylated CpG motifs (Britigan, Lewis et al. 2001). Lactoferrin is cleaved by cystinyl cathepsins in the CF lung and this undoubtedly has a myriad of pro-inflammatory effects (Rogan, Taggart et al. 2004) .

1.3.8 Lysozyme

Lysozyme, termed a cell wall degrading enzyme is another important component of the lung's anti-microbial array. It possesses the ability to disrupt the β1→4 glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid residues that make up peptidoglycan, the cell wall component of bacteria (Fett, Strydom et al. 1985). Whilst sharing some properties with lactoferrin, it importantly does not have any anti-viral or anti-inflammatory properties.

1.4 Anaerobic Bacteria

To date, the study of anaerobic bacteria has been limited, partially due to the fact that the standard anaerobic culture methods are quite labour intensive and unreliable (Goldstein, Citron et al. 1992). Nucleic acid amplification of 16S rRNA genes specific for the domain bacteria followed by postamplification analyses using terminal restriction fragment length (t-RFLP), and Sanger sequencing have been used to characterise CF sputum.(Rogers, Carroll et al. 2004, Harris, De Groote et al. 2007, Bittar, Richet et al. 2008, Armougom, Bittar et al. 2009, Bittar and Rolain 2010). Molecular detection techniques are now becoming more common and are more sensitive than culture methods at detecting anaerobic bacteria. Using quantative PCR methods anaerobes have been found to be as prevalent in CF sputum as typical CF pathogens(Zemanick, Wagner et al. 2010). This is a diverse population of microorganisms with eight bacterial phyla, comprising 460 genera, including facultative and obligate anaerobes, oral bacteria and opportunistic pathogens, many of which have never before been identified in the CF lung (Guss, Roeselers et al. 2011).

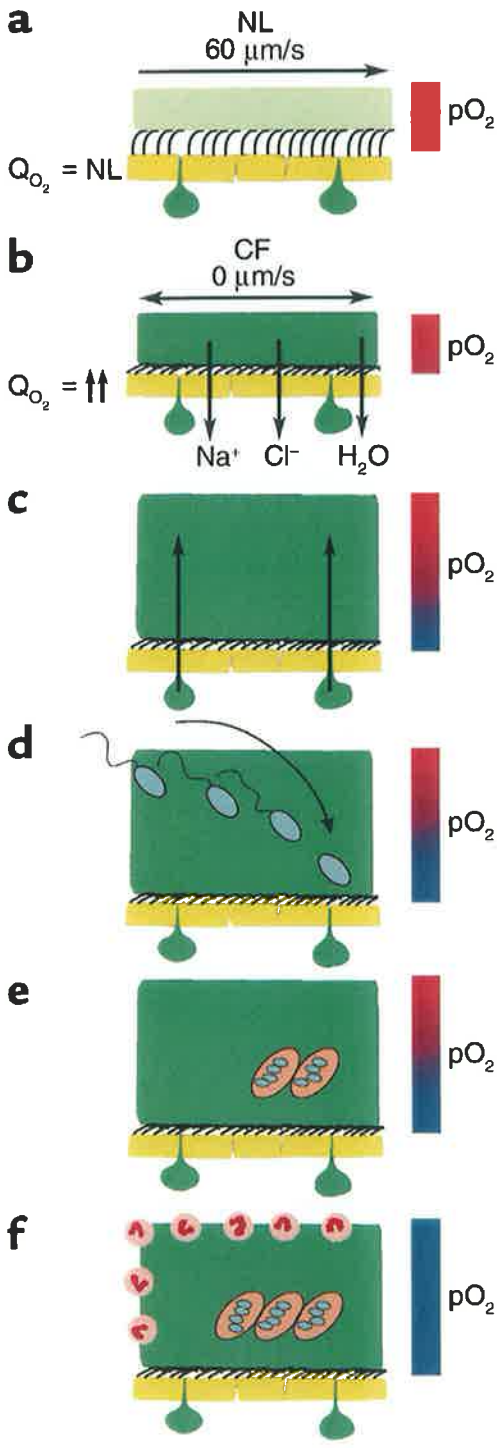


Figure 1.3: Schematic model of the pathogenic events hypothesized to lead to chronic Pseudomonas aeruginosa infection in airways of CF patients.
(a) On normal airway epithelia, a thin mucus layer (light green) resides on top of the peri ciliary layer (PCL) (clear). The presence of the low-viscosity PCL allows

efficient mucociliary clearance (denoted by vector). A normal rate of epithelial O2 consumption (QO2; left) produces no O2 gradients within this thin airway surface liquid (ASL) (denoted by red bar). (b-f) CF airway epithelia. (b) Excessive CF volume depletion (denoted by vertical arrows) removes the PCL, mucus adheres to epithelial surfaces, and mucus transport slows/stops (bidirectional vector. The raised CF oxygen consumption generates steep hypoxic gradients (blue colour in bar) in thickened mucus masses. (d) P. aeruginosa bacteria deposited on mucus surfaces penetrate actively and/or passively (due to mucus turbulence) into hypoxic zones within the mucus masses. (e) P. aeruginosa adapts to hypoxic areas within mucus and with increased alginate formation this promotes the creation of macrocolonies. (f) Macrocolonies resist neutrophils, causing chronic infection. The presence of neutrophils, encourage hypoxic conditions (blue bar). (Adapted from Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. Doring G et al. J. Clin. Invest.(2002); 109:317-325

Common aerobic bacteria in the CF lung can also exist anaerobically by the above proposed mechanism (Figure 1.3).Anaerobic bacteria have been cultured from 69% of samples where *P. aeruginosa* was also isolated and in 41% of samples where it is not, *Prevotella, Veillonella*, *Propionibacteria* and *Actinomyces* were the most commonly identified. Studies in CF patients have shown that bacteria from oral mouth wash specimens and sputum specimens are not similar suggesting that anaerobes in CF are not contaminates but originate from the lower respiratory tract (Tunney, Field et al. 2008, Worlitzsch, Rintelen et al. 2009, Jones 2011). Sensitivity testing produces diverse results and in these studies it has been shown that isolates of *Propionibacterium* are resistant to metronidazole, isolates of *Veillonella* are resistant to piperacillin/tazobactam and some isolates of *Prevotella* are resistant to ampicillin, clindamycin and metronidazole. Another study from Sherrard and colleagues(Sherrard, Graham et al. 2013) found that for prevotella species in CF, meropenem, chloramphenicol, piperacillin/tazobactam and metronidazole are likely to be the most effective

antibiotics. On studying patients pre and post exacerbation, (Fodor, Klem et al. 2012) it was noted that there was very little difference in the microorganisms detected raising the question as to the role that anaerobes play in the CF airway as a pathogen or if they are "innocent bystanders" in respect of an active inflammatory role of anaerobes. However, Zemanick and colleagues, published recently (Zemanick, Harris et al. 2013), that anaerobes identified from sputum by sequencing during pulmonary exacerbations of CF are associated with less inflammation and higher lung function compared to Pseudomonas at early exacerbation. On the other hand, a recent study has suggested that oral bacteria and anaerobes are more frequently detected in community acquired pneumonia than previously thought in the non-CF population (Yamasaki, Kawanami et al. 2013), implying that their presence could still have implication in possibly increasing pathogenicity.

Many anaerobic bacteria that colonise CF airways have been shown to produce different potent virulence factors. The proteolytic activity of bacteria has been the focus of many studies because such activity is associated with the local degradation of connective tissue as well as the breakdown of proteinaceous molecules associated with the host defense mechanisms (Eley and Cox 2003). Within the *Prevotella* genus, *P. intermedia* and *P. nigrescens* have previously been reported to exhibit proteinase activity (Eley and Cox 2003). However, there is comparatively little information regarding the proteolytic activity of the other *Prevotella* species frequently recovered from sites of oral infection (Yanagisawa, Kuriyama et al. 2006). *P.intermedia* is associated with severe periodontal disease, and has been found in CF sputum. In *P. intermedia*, several proteases

have been described, among them trypsin-like serine proteases, a dipeptidyl peptidase IV and cysteine proteases (Shibata, Miwa et al. 2003, Guan, Nagata et al. 2006). Interpain A, a cysteine proteinase from *P. intermedia*, inhibits complement by degrading complement factor C3 (Potempa, Potempa et al. 2009). Interpain A is also involved in the breakdown and release of haem from haemoglobin, which is itself pro-inflammatory (Byrne, Wawrzonek et al. 2009). Mallorqui- Fernadez and colleagues have described a new autocatalytic mechanism on Interpain A which provides more proteolytic mechanisms (Mallorquí-Fernández, Manandhar et al. 2008).

1.4.1 Prevotella melaninogenica

P. melaninogenica a gram-negative, anaerobic, rod-shaped, pigment-producing bacterium is found both in the oral cavity and in the CF lung. *P. melaninogenica* is thought to exert virulence at least in part through haemolysis and haemagglutination. HA of *P. melaninogenica* seems to be mediated through a lectin-like protein, binding to raffinose, lactose and galactose-containing carbohydrate residues on erythrocytes.It would appear that *P. gingivalis* and *P. intermedia* are more pathogenic through this mechanism than *P. melaninogenica* (Haraldsson, Meurman et al. 2005).

There is evidence that MMP-9 also increases during chronic inflammation (Tayebjee, Tan et al. 2005). *P. gingivalis, P. inter-media, P. nigrescens, Fusobacterium nucleatum (F. nucleatum)* can all produce gelatinolytic proteinases which, in turn, may activate latent proMMP-9. This may suggests a co-operative cascade in the pathogenesis of oral and perhaps lung tissue destruction. Synthetic proteinase inhibitors such as MMP inhibitors and pefabloc

only exhibit slight inhibitory effects on the proteolytic activities of these bacteria (Jie Bao, Kari et al. 2008).

1.4.2 *Prevotella intermedia*

Complement is an important host defense mechanism against *P. intermedia.* The different strains of *P. intermedia* show variable sensitivity to the complement-dependent bactericidal activity of serum. The alternative pathway was shown to contribute to the killing of serum sensitive strains, while the classical pathway was primarily responsible for killing strains with intermediate sensitivity (Krauss, Potempa et al. 2000, Potempa, Potempa et al. 2009) Interpain A (InpA) a cysteine protease produced by *P. intermedia* was 100% resistant to killing (Potempa, Potempa et al. 2009). InpA can activate complement C1 complex in serum even at low concentrations and this causes C1q deposition on bacterial surfaces resulting in a local inflammation. Initially InpA can release C3a and the complement cascade, at high concentrations they can be suppressed, inhibiting complement activation and increase biofilm formation (Potempa, Potempa et al. 2009).

1.4.3 *Fusobacterium nucleatum*

F. nucleatum can cause death of human lymphocytes and cause endothelial injury via FadA adhesin which binds vascular endothelin cadherin (Fardini, Wang et al. 2011). This is cited as a reason why this bacterium is involved in multiple infections and is able to disseminate so widely. It also has been shown to increase its invasive potential by acquiring MMP-9 activity (Gendron, Plamondon et al. 2004). Most oral pathogens which are largely

anaerobic bacteria favour TLR 2 activation rather than TLR4 immune response (Kikkert, Laine et al. 2007) . Anaerobic bacteria produce short chain fatty acids (SCFA) as by-products of fermentation when these SCFA interact with TLR2 ligand, it can cause IL-8 and TNF α release (Mirmonsef, Zariffard et al. 2012). Toussi et al (Toussi, Liu et al. 2012) indicated that the FomA porin from *F. nucleatum* has immune adjuvant activity in mice. FomA increases interleukin 8 (IL-8) secretion and NF-κB-dependent luciferase activity in HEK cells expressing TLR2. In a mouse model of immunization with ovalbumin (OVA), FomA induces enhanced production of OVA-specific IgM and IgG, and enhanced secretion of IL-10 and IL-6, consistent with a Th2-type effect. Anti-FomA antibodies are also produced, suggesting that FomA is immunogenic, which is TLR2 dependent.

1.4.4 *Streptococcus milleri*

Streptococcus intermedius, *Streptococcus anginosus*, and *Streptococcus constellatus* are a group of organisms collectively referred to as the *Streptococcus milleri* group (SMG) or the *S. anginosus* group. *S.milleri* has now been described in CF as being a potential pulmonary pathogen (Parkins, Sibley et al. 2008, Sibley, Parkins et al. 2008). The SMG has been shown previously to be are linked with clinical deterioration in 40% of hospital admissions in a CF cohort (Sibley, Parkins et al. 2008). Members of the SMG have shown that these microorganisms are phenotypically similar and that several strains produce different virulence factors, such as hydrolytic enzymes and hemolysins (Jacobs and Stobberingh 1995) (Whiley, Fraser et al. 1990, Grinwis, Sibley et al. 2010) Hydrolytic enzymes are able to break down host tissue and are the probable

virulent factors involved. The SMG group did produce proteases, however not all groups have found similar findings (Ruoff and Ferraro 1987).

1.4.5 Anaerobic metabolism

Under aerobic conditions, most bacteria will oxidize glucose and other saccharides to pyruvate, and complete the Krebs cycle. These both require oxygen as the final electron acceptor. Under anaerobic conditions, bacteria must use an alternate electron acceptor (e.g. sulfur, nitrates or carbon dioxide), use the energy of another biochemical reaction, or bypass oxidative respiration (Zumft 1997). The process of fermentation involves the release of energy from compounds without use of exogenous oxygen. Fermentative processes also occur in eukaryotes, such as in muscle cells during exercise through the formation of lactic acid. *Pseudomonas* species have a set of enzymes that can reduce nitrate to nitrite, nitric oxide, nitrous oxide, and finally nitrogen gas (Figure 1.4).

In the context of CF lung infections, *P. aeruginosa* nitrate metabolism has been largely associated with its anaerobic metabolism (Yoon, Hennigan et al. 2002, Hassett, Sutton et al. 2009, Schobert and Jahn 2010). In normoxia NADH is generated by glycolysis and the tricarboxylic acid (TCA) cycle. In hypoxia NADH is also generated through glycolysis and the TCA cycle,with the addition of the glyoxylate shunt. The glyoxylate shunt is a bypass of the TCA cycle, avoiding the steps which require O2 and release CO2.

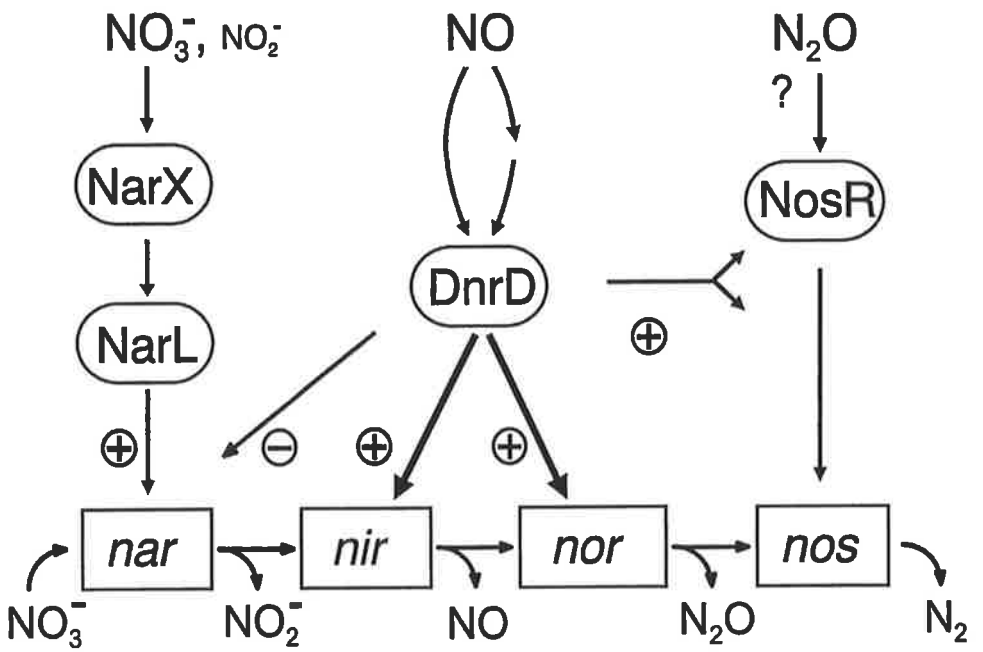


Figure 1.4: A method by which P. stutzeri undergoes anaerobic metabolism by a process known as denitrification.

1.5 Short Chain Fatty Acids

Short chain fatty acids (SCFAs) are small carboxylic acids containing 2 to 6 carbon atoms. They are the products of colonic bacterial degradation of unabsorbed starch and non-starch polysaccharide (fibre). The most abundant SCFAs are the C2-C4: acetic acid, propionic acid and butyric acid. SCFAs also include C4 and C5 derivatives iso-butyrate, succinate, valerate, and iso-valerate, which are found in relatively lower abundance compared to the smaller SCFAs. These molecules have a pKa around 4.8, which means that at neutral pH they are almost 99% ionized and thus the anionic species is predominant(Levison 1973). At pH 6.0, roughly 6% of SCFA species will be protonated. SCFAs are produced primarily in the colon by commensal gut bacteria through anaerobic fermentation of undigested carbohydrates and amino acids. Acetate (C2), propionate (C3) and butyrate (C4) are found in the human intestine at concentrations of approximately 13 mM in the terminal ileum, ~130 mM in the caecum and ~80 mM in the descending colon (Cummings, Pomare et al. 1987). SCFAs released in the intestinal lumen are readilyabsorbed and used as energy source by colonocytes (5 to 10% of human basal energy requirements are provided by SCFAs) and also by other tissues including liver and muscle(McNeil , Cummings N.H. et al. 1978).

The function of SCFAs in the lung is largely unknown. Eftiamiadi et al. described that the phagocytosis of *S.aureus* by lung phagocytes was strongly inhibited by the end products of anaerobic catabolism, namely butyric, propionic and succinic acids and that these anti-phagocytic effects were dependent on presence of these SCFAs (Eftimiadi C 1990). The other role or function of SCFAs

specifically in the lung is as of yet unknown about and much of the data is extrapolated from gastrointestinal studies.

In general, SCFA are produced by metabolism of pyruvate, which is produced by oxidation of glucose through glycolysis (Miller and Wolin 1996). In the gut, SCFAs have a complex set of roles, being involved in energy provision, mediating inflammation, regulating cell proliferation and apoptosis, hormone secretion(Plaisancié, Dumoulin et al. 1996) regulating immune responses (Maslowski, Vieira et al. 2009) and gene expression through histone deacetylation inhibition activity (Vinolo, Rodrigues et al. 2011) . In the intestine, SCFA stimulates water and electrolyte uptake. SCFA increases colonic blood flow and have a trophic effect on colonic epithelium (Figure 1.6)(Scheppach 1994).

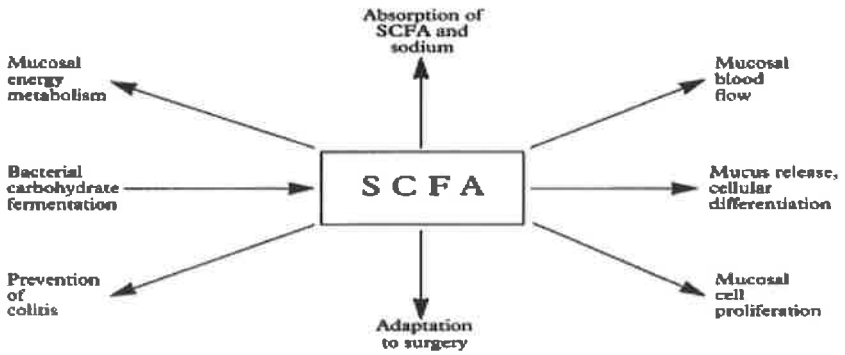


Figure 1.5: Functions of SCFAs in the Gut
Adapted from (Scheppach 1994)

1.5.1 Metabolism of SCFA

The method on how the gut breaks down carbohydrates into SCFA has been well described. SCFA production requires that the gut microbiota work

together with the host. The H_2 that is produced during acetate formation can be used by other bacteria to avoid accumulation of H_2 which wouldn't allow fermenters to oxidise NADPH. The CO_2, required for electron transfer, is provided by the host and it is excreted into the lumen of the gut as HCO_3^- in exchange for SCFA anions. This is an important step as a pH regulatory mechanism because protons in the lumen of the gut, formed during the production of SCFA are neutralised by bicarbonate under the formation of CO_2 (den Besten, van Eunen et al. 2013). The production of acetate, butyrate and propionate from carbohydrates are described below. (Figure 1.6, 1.7) In the gut, there is a drop of pH from ileum and caecum, and this changes gut flora as it is transitioned through the bowel, for example in the distal large intestine, the pH is 6.5 and so butyrate producing bacteria disappear and bacteria that produce acetate and propionate, namely bacteroides appear (Walker, Duncan et al. 2005).

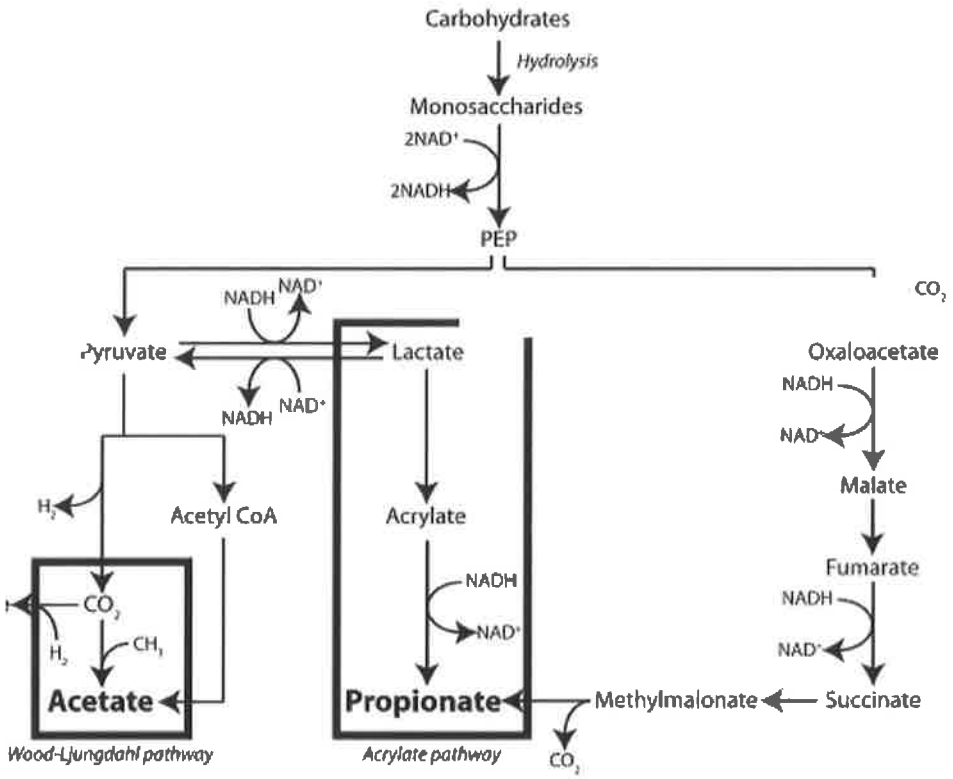


Figure 1.6: Overview of production of acetate and propionate from carbohydrates. H+ produced during acetate formation is used by other bacteria working closely in the community. If this is not done, NADPH is not metabolised. The CO2 required is provided by the host. This is used as an important pH regulatory system in the gut; it is primarily obtained from HCO3- in exchange for SCFA anions. Fibre high in SCFA lower the pH in the colon and so affect the colon microbiome and production of SCFA.PEP - phosphoenol pyruvate. Adapted from (den Besten, van Eunen et al. 2013)

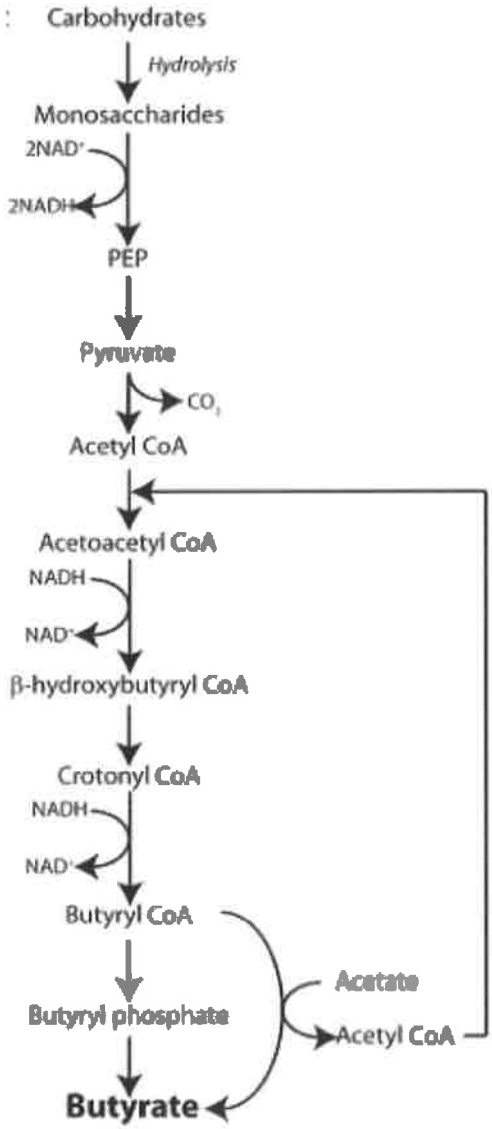


Figure 1.7: Overview of the production of butyrate from carbohydrate production
Adapted from (den Besten, van Eunen et al. 2013)

1.5.2 Receptors for SCFA

The family of G-coupled protein receptors (GPR) are known to recognise fatty acids. Free fatty acid receptors 1 (FFAR1/GPR40), 2 (FFAR2/GPR43), and 3 (FFAR3/GPR41), GPR84, and GPR120 have different specificities for fatty acids. FFAR1/GPR 40 is the receptor for medium and long chained fatty acids. GPR84 recognizes medium-chain fatty acids (capric acid, undecanoic acid, lauric acid) with EC50s in the 5 to 10 μM range (Wang, Wu et al. 2006) GPR 41 and GPR 43 have been deorphanised (Brown, Goldsworthy et al. 2003, Le Poul, Loison et al. 2003, Nilsson, Kotarsky et al. 2003). Acetate and propionate were found to be the two most potent ligands, although butyrate, formate, and valerate (in this order of potency) also were able to induce receptor activation, these activated receptors by high micromolar concentrations of SCFA(Topping and Clifton 2001). GPR 41 has a higher affinity for longer SCFA, and GPR 43 has higher potency for formic acid (C1) and acetic acid (C2). The potency rank order of SCFAs for GPR 43 is acetate (C2) - propionate (C3) > butyrate (C4) > valerate (C5) > formate (C1). The rank order for GPR 41 is propionate(C3) -butyrate(C4) - valerate(C5) > acetate(C2) > caproate(C6).(Brown, Goldsworthy et al. 2003, Le Poul, Loison et al. 2003).

1.5.3 GPR 43/FFAR 2

GPR 43 is known to be highly expressed in immune cells such as monocytes, granulocytes and lymphocytes, as well as enteroendocrine cells and neutrophils (Brown, Goldsworthy et al. 2003, Le Poul, Loison et al. 2003, Nilsson, Kotarsky et al. 2003). Several studies have demonstrated that GPR 43 mediates the chemotactic effects of SCFAs on neutrophils (Le Poul, Loison et al. 2003, Maslowski, Vieira et al. 2009, Sina, Gavrilova et al. 2009, Vinolo, Rodrigues et al. 2011) . A high fiber diet is associated with decreased risk of inflammatory bowel diseases (Hou, Abraham et al. 2011). Sina and colleagues found that GPR 43 deficient-mice showed unresolving or worsening colitis, and also reduced inflammatory response and reduced PMNs .These results indicate that antagonists of GPR 43 could have a role in treatment of intestinal inflammation (Sina, Gavrilova et al. 2009). GPR 41 and 43 are both expressed in the intestine and colocalize with a subset of enteroendocrine cells in the mucosal epithelium that express Peptide YY (PYY) (Karaki, Mitsui et al. 2006, Tazoe, Otomo et al. 2009). FFAR3 deficiency in mice was associated with an attenuated microbiota-induced increase in plasma PYY (Samuel B.S., Shaito et al. 2008). Enteroendocrine L cells also can secrete GLP-1 secretion, an anoretic hormone which also regulates insulin secretion (Karaki, Mitsui et al. 2006) . SCFAs activate GPR43 with low sensitivity, the EC50 for acetate being ~430microM, and for butyrate and propionate ~300microM for GTP gammaS binding, this is different to the levels in the serum. Butyrate has been known to have antitumour effects, and evidence suggests that other SCFAs might prevent colon carcinogenesis. Thus, Tang and coworkers (Tang, Chen et al. 2011) tested

whether the effects of SCFAs were mediated by GPR43. Using specific antibodies, immunoreactivity for GPR43 was found in the normal human colonic epithelium but was markedly reduced or absent in colon cancer.Propionate treatment of GPR43-restored cells, induced p21 expression and decreased cyclin D3- and cyclin-dependent kinases 1 and 2. The data is consistent with a tumor suppressor function of luminally derived SCFAs and is a possible mechanism for lower rates of colonic cancer with a high fibre diet. GPR 43 is able to set up further signalling effects downstream as described below (Figure 1.8).

1.5.4 GPR 41/FFAR 3

GPR 41 is another receptor for SCFAs with a slightly different specificity. GPR 41 is expressed in enteroendocrine cells, sympathetic ganglia, and pancreatic β-cells, PBMC, spleen and adipose tissue (Brown, Goldsworthy et al. 2003, Le Poul, Loison et al. 2003). GPR 41 expression found in mouse and human adipose tissue, and SCFA through the receptor induce leptin production (Xiong, Miyamoto et al. 2004). GPR 41 recognizes propionate, butyrate, valerate and caproate, but has much weaker affinity for acetate (Le Poul, Loison et al. 2003). In contrast with GPR 43 which has similar affinities for propionate and acetate, the EC50s of propionate and acetate for GPR 41 are between 0.63 μM to 20 μM and 1 mM, respectively(Le Poul, Loison et al. 2003). GPR 41 is required for maximal GLP-1 induction by butyrate(Lin, Frassetto et al. 2012) and GPR 41expression on enteroendocrine cells could be involved in PYY release(Karaki, Mitsui et al. 2006) . SCFAs and ketone bodies regulate the sympathetic nervous system through GPR 41.β hydroxybutrate a metabolite produced during the

starvation state antagonise GPR 41 and reduce the sympathetic nervous system activity (Kimura, Inoue et al. 2011) .

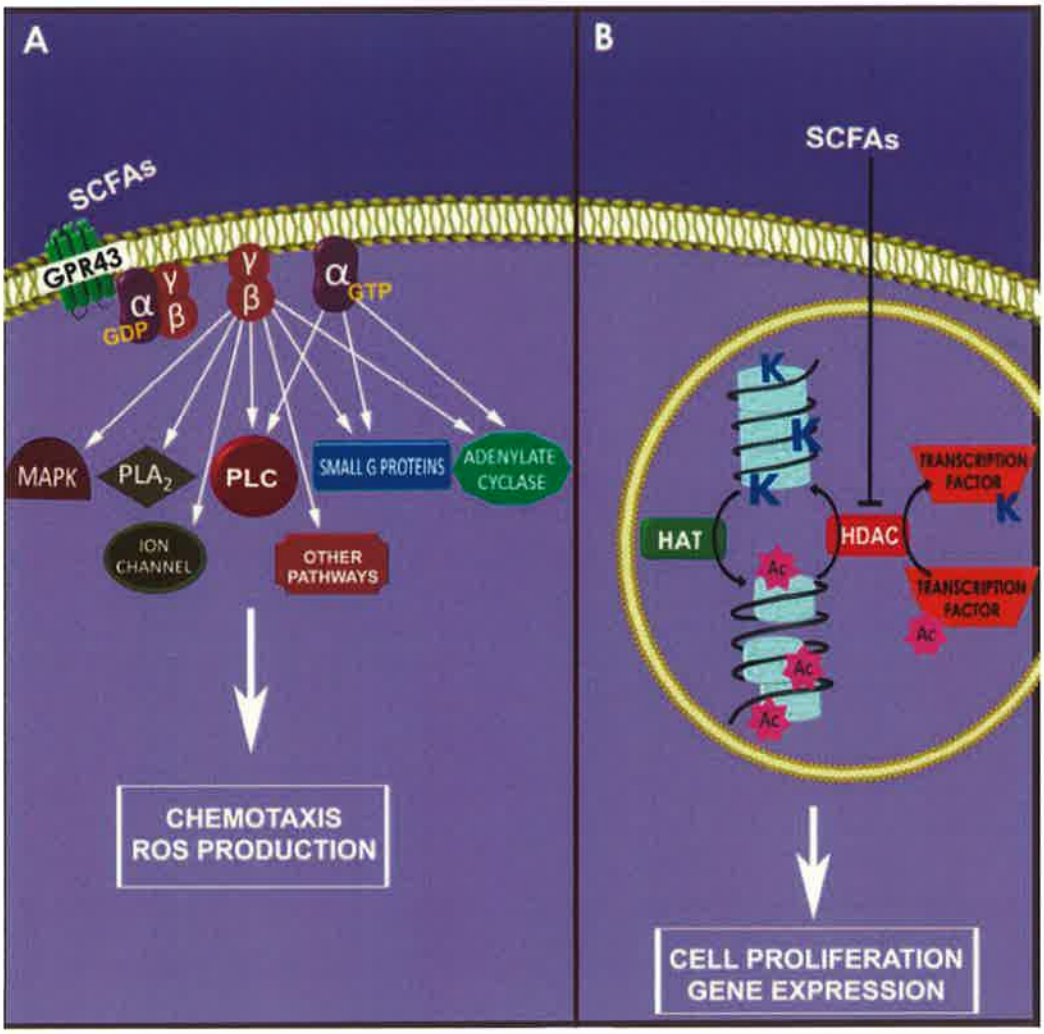


Figure 1.8: Overview of the signalling pathways activated downstream of GPR43 receptors and representation of the effects of SCFAs through inhibition of histone deacetylase (HDAC) activity.

GPR43 couples to Gi and Gq proteins, which interact with several proteins including adenylate cyclase, small G proteins (e.g., Rac and Rho),mitogen-activated protein kinases (MAPK), phospholipase C (PLC) and A2 (PLA2), ionchannels and transcription factors (**A**). SCFAs may also act on cells through inhibition of HDAC (**B**). Adapted from (Vinolo, Rodrigues et al. 2011)

1.5.5 Effect of SCFA on inflammation

SCFAs can cause chemotaxis of neutrophils *in vitro* by activating GPR43 (Le Poul, Loison et al. 2003, Sina, Gavrilova et al. 2009, Vinolo, Ferguson et al. 2011). GPR43 couples to Gi/oand Gq proteins (Brown, Goldsworthy et al. 2003, Le Poul, Loison et al. 2003, Nilsson, Kotarsky et al. 2003). Binding of agonists to this receptor activates several intracellular pathways including mitogen-activated proteinkinases (MAPKs), protein kinase C and transcriptional factors such as activating transcriptionalfactor-2, also known as ATF-2 (Maslowski, Vieira et al. 2009). SCFAs through GPR43 cause activation of PKB and MAPKs (p38 and ERK) in neutrophils. These responses are sensitive to pertussis toxin treatment, indicating a role for Gi proteins (Vinolo, Ferguson et al. 2011). Chemotaxis of neutrophils induced by formyl-Met-Leu-Phe (fMLP) or KC (CXCL1), a chemokine for mouse neutrophils, can be either increased (Sina, Gavrilova et al. 2009) or not affected by SCFAs (Vinolo, Ferguson et al. 2011). SCFAs also reduce the surface expression of chemoattractant receptors (e.g., C5aR and CXCR2). Butyrate can regulate and release IL-8, MCP-1 and CXCL-1 by intestinal epithelial cells in response to cytokines(Bocker, Nebe et al. 2003). In human colonic subepithelial myofibroblasts, butyrate, blocks the expression of interferon (IFN)-gamma-inducible protein-10 (IP-10, CXCL-10), a chemoattractant for T lymphocytes and monocytes (Inatomi, Andoh et al. 2005).Activated macrophages produce TNF-α, IL-1β and IL-6, chemokines, nitric oxide (NO) and arachidonic acid derivatives. SCFAs, mainly butyrate, suppress the LPS- and cytokine- production of pro-inflammatory mediators including TNF-α, IL-6 and NO. Butyrate also enhances the release of the anti-inflammatorycytokine IL-10.

However, this latter effect was not found in all studies; for example, Cox *et al.*, instead of an increase, found an attenuation of IL-10 production by monocytes treated with SCFAs (Cox, Jackson et al. 2009).SCFA activating GPR 41 and 43 can cause increase cytokine production by MAPK signalling and cause tissue inflammation (Kim, Kang et al.). SCFA can cause reduce HDAC activity and butyrate is the most potent for this. If HDAC is inhibited, SCFAs can increase acetylation of histone and other proteins for example NKκB, MyoD,p53 and N-FAT (Glozak, Sengupta et al. 2005). SCFAs activate GPR41 and GPR43 on intestinal epithelial cells, leading to MAPK signalling and rapid production of chemokines and cytokines. These pathways mediate protective immunity and tissue inflammation in mice (Kim, Kang et al. 2013).

SCFA can cause release of PGE_2 by human monocytes, this prostaglandin was considered to be anti-inflammatory as it can reduce TNF α and IL-β by macrophages and reduce Th-1 differentiation. However, it has been shown that through its EP 4 receptor, it permits Th1 differentiation and Th-17 expansion (Yao, Sakata et al. 2009). Other anti-inflammatory roles are seen in neutrophils, when, acetate, propionate and butyrate reach 30mM there is a reduced TNF α production by LPS mediated neutrophils (Tedelind, Westberg et al. 2007). Both inhibition and stimulation of neutrophil phagocytosis by SCFAs have been described (Eftimiadi, Tonetti et al. 1990). SCFA can influence lymphocytes that are important in the adaptive immunity. They can cause T cell proliferation, production in IL-2 response and produce regulatory T cells, which all work in actively suppressing immune response (Cavaglieri, Nishiyama et al.

2003, Tao, de Zoeten et al. 2007). Inflammatory effects by SCFA is described below (Table 1.1)(Vinolo, Rodrigues et al. 2011).

Cell type	Effect seen	SCFA	Reference
Mononuclear cells	↓TNF α ↑PGE_2	Butyrate	(Usami, Kishimoto et al. 2008)
Macrophages	↓TNF α	Butyrate	(Fukae, Amasaki et al. 2005)
Monocytes	↓TNF α, IL-12, IFN-α ↑IL-10 ↓MCP-1, IL-10, ↑PGE_2	Butyrate Acetate, Butyrate, Proprionic Acid	(Saemann, Bohmig et al. 2000)

Table 1.1: Inflammatory mediators produced by SCFA

1.5.6 SCFAs in infection

Different methods of measuring SCFA in anaerobic bacteria have been described by previous authors (James and Martin 1952, Guerrant, Lambert et al. 1982, Tangerman and Nagengast 1996, Pouteau, Meirim et al. 2001, Harrison 2010) .Gas or gas-liquid chromatography (GC or GLC) was first used to diagnose anaerobic infections in abscesses and empyema(Gorbach, Mayhew et al. 1976, Ladas, Arapakis et al. 1979). The main benefit of GC analysis of SCFAs is the rapid test which has good clinical correlation with culture of anaerobic bacteria. Botta et al. (Botta, Radin et al. 1985) found that gingival inflammation, correlated positively with SCFA concentrations, especially isobutyrate, butyrate, and isovalerate. The presence of SCFA in clinical specimens suggests the presence of anaerobic infection.

1.6 Rationale

Currently, there are over 30,000 CF patients in the United States, 8000 in the United Kingdom and 1400 in Ireland. The median survival of CF patients is ~37 years (McCormick, Green et al. 2002), with chronic bacterial pulmonary infection and recurring pulmonary exacerbations ultimately producing irreversible decline in lung function, respiratory failure, and death . Lung disease results from the host inflammatory response to bacterial infection (Ramsey 1996). However, these bacteria also produce extracellular enzymes such as proteases, which may produce bronchiectatic changes in the airway wall by digesting structural proteins and peptides importantin innate immunity (Suter 1994).

Standard microbiological identification of the bacterial microbiota in sputum, and antibiotic susceptibilitytesting guides antibiotic treatment during pulmonary exacerbations. The bacteria most frequently isolated from the sputum of CF patients by those methods are *S. aureus* and *Haemophilus influenzae (H. influenzae)* in the early years of life with *P. aeruginosa* and *Burkholderia cepacia (B. cepacia)* complex common in older patients (Grasemann, Ioannidis et al. 1998).

There are steep oxygen gradients within mucus in CF lungs, and proliferation of *P. aeruginosa* within mucus creates hypoxic (< 2 Torr O2) conditions (Worlitzsch, Tarran et al. 2002).Consistent with this observation, the proliferation of *P. aeruginosa* under batch or continuous culture conditions results in the rapid reduction of O2 tension within the media (Kim, Wang et al. 2005). *P. aeruginosa* can respire utilizing molecular O2 at ~2 Torr sufficiently well to proliferate (Alvarez-Ortega and Harwood 2007) and can use nitrite and nitrate at

concentrations present in CF airway surface liquid (ASL) (Hassett 1996) as alternate electron acceptors, allowing growth in strictly anaerobic environments (Hassett, Cuppoletti et al. 2002). *P. aeruginosa* also grows well as biofilms under anaerobic conditions (Yoon, Hennigan et al. 2002).

Molecular analysis by 16S rRNA sequencing has shown that there are over 700 species of bacteria in the mouth (Paster 2006). Oral bacteria have evolved to form biofilms on hard tooth surfaces and on soft epithelial tissues. Initial colonisers of the tooth pedicle include *Streptococcus sanguinis* (*S. sanguinis*), *Streptococcus gordonii, Streptococcus mitis, Streptococcus oralis* and *Actinomyces* species, these bind to complementary salivary receptors (sialylated mucins, proline-rich protein, α amylase, salivary agglutinin and bacterial cell fragments) on the tooth surface. Middle and late colonisers are able to adhere via coadhesion and coaggregation mechanisms and bacteria of the genus *Fusobacterium* exhibit more links than any other bacteria and are able to form important bonds between all the colonisers. These form the cornerstone of the building of the biofilm (Figure 1.9)(Kolenbrander, Palmer et al. 2010).In CF patients it is unusual to isolate anaerobes in routine hospital culture and in a large study by our collaborators 64% of sputum samples taken from patients with CF, which were specifically grown for anaerobes, were colonised with primarily *Prevotella* and *Veillonella* strains (Tunney, Field et al. 2008). The colonisation of *P.aeruginosa* significantly increased the likelihood that anaerobic bacteria would be present in CF sputum (Tunney, Field et al. 2008). Recent data demonstrate that the oropharyngeal flora, that are not normally thought of as serious problems in CF, can induce a positive regulation (phenomenon of quorum sensing) of

genes of virulence of *P. aeruginosa* (Duan, Dammel et al. 2003). It is also possible that *P. aeruginosa* can influence virulence factor genes of those potentially present in the oropharyngeal flora strains that gain in pathogenicity (Duan, Dammel et al. 2003). Therefore, oropharyngeal bacteria likely participate in disease progression and are probably an underestimated emerging cause of CF lung pathology that should be considered in the antibiotic strategy. New therapeutic approaches by inhibition of the phenomenon of quorum sensing and anaerobes may be useful in the treatment of the respiratory infections in CF patients as recently demonstrated with azithromycin (Hoffmann, Lee et al. 2007).

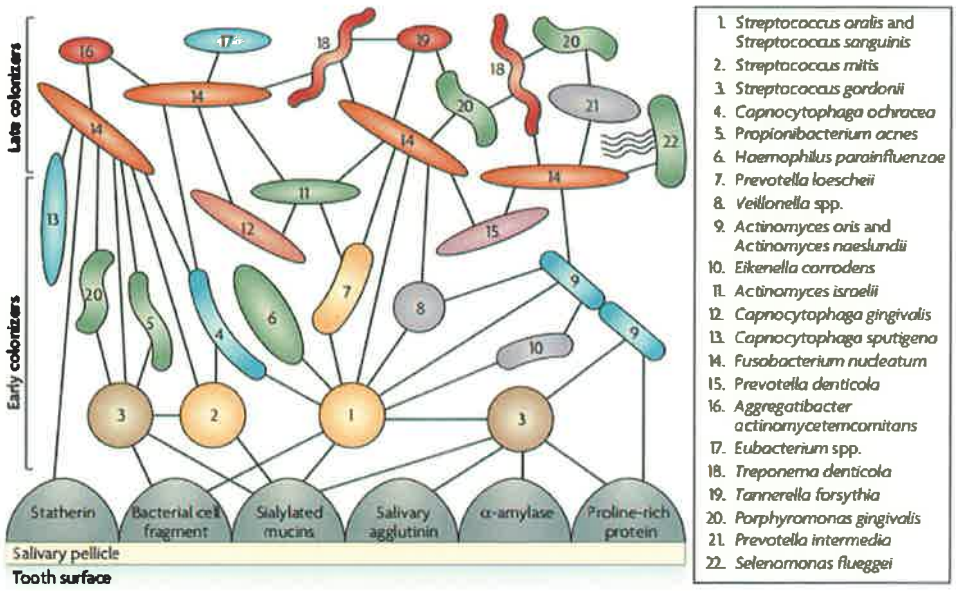


Figure 1.9: Model of oral bacterial colonisation.
Initial colonising bacteria, *Fusobacterium* and late colonisers recognise the salivary pedicle receptors on the tooth surface. These interactions represent the development of a dental plaque. Adapted from (Kolenbrander, Palmer et al. 2010)

As anaerobic conditions exist in the lungs of CF patients with persistent respiratory infection (Worlitzsch, Tarran et al. 2002), we hypothesized that anaerobic bacteria, not detected by routine aerobic culture methods, are present as polymicrobial or "mixed" CF pulmonary infections. Anaerobic bacteria are found in CF sputum and BAL, however the clinical significance of these bacteria remain undetermined. Patients with CF get regular exacerbations; however, they don't specifically receive treatment for anaerobic infections. This study is part of a

multi centred trial with Queen's University Belfast and University of North Carolina. The aim of the study is to recruit 450 patients with CF both children and adults. Initially, the cross sectional group would provide a sputum sample, induced sputum sample or BAL sample together with urine and plasma and serum samples. 150 patients will be followed up in the longitudinal group and a clinical sample will be obtained both at stable and at pre and post exacerbation time points in both adults and children. This is important to establish to see if the anaerobic infection represents the natural micro flora of the lung or if it is pathogenic and could be responsible for infections. Anaerobic infections could be pathogenic both by the production of short chain fatty acids, which can influence the inflammatory process and by the production of anaerobic proteases, which can inhibit the innate human defensins such as AAT, SLPI, elafin, lactoferrin and human defensins.

1.7 Hypothesis

The present proposal tests the hypothesis that the hypoxic environment of the CF airways promotes infection by obligate anaerobes as mixed infections that are pathogenic for the CF lung. Anaerobic bacteria in CF sputum and BAL is prevalent, certain anaerobic bacteria are pathogenic and produce proteases and short chain fatty acids. These anaerobic bacteria are present in exacerbations and specific antibiotic targeted therapy should be used at the time of exacerbation in order to eradicate infection.

1.8 Aim

The aim of the project was to detect the presence of anaerobic bacteria by routine culture and second generation sequencing in CF patients across all age ranges and during stable and exacerbation episodes. Following this, we identified the bacteria most prevalent in this population group and we further proposed to investigate if the bacteria found were pathogenic. In our laboratory, we had previously studied bacterial proteases from aerobic bacteria and now we wished to investigate, if anaerobic bacteria produced proteases and if they interfered with the innate human immune system. Secondly, by- products of anaerobic bacteria, SCFA, have previously been investigated in the gastrointestinal tract, however anaerobic bacteria in the lung was a novel concept and we wish to further investigate to see if they had a pathogenic role.

Materials and Methods

Chapter 2

2.1 Recruitment

450 CF patients cross-sectionally were recruited across 3 sites, Dublin (Beaumont hospital, Our Lady's Children's' hospital, Crumlin and Children's' University hospital, Temple Street), University of North Carolina (UNC) and Queen's University Belfast (QUB) and 150 patients totally longitudinally.CF patients were diagnosed according to published guidelines (Flume, Mogayzel et al. 2009) and were recruited from out-patient clinics at UNC-Chapel Hill, Belfast, and Dublin. Consent was obtained as per local ethics committee requirements (Appendix 8.1-8.10). Ethics was obtained in Dublin from Beaumont hospital, Our Lady's Children's' Hospital for Sick Children, Crumlin and Children's' University Hospital ethics committees. The cross-sectional approach was to stratify CF patients into 3 age groups: 0-6 years, >6-18 years and >18 years. In the 0-6 age group, BAL was obtained using excess material from CF children undergoing clinically indicated procedures. BAL was performed as per clinical routine using a laryngeal mask airway (LMA) or endotracheal tube (ETT) which forms a low pressure seal around the entrance to the larynx, protecting the trachea from pharyngeal secretions. Bronchoscopy was routinely performed at two of our study sites. At UNC, bronchoscopy was performed at times of CF respiratory exacerbation in children who could not expectorate or when *P. Aeruginosa* was isolated for the first time from a deep pharyngeal throat culture. In Dublin, annual bronchoscopies are done to assess infection and airway inflammation.

Hypertonic saline induction was used for all control subjects. All samples were immediately divided; one aliquot was placed in an anaerobic jar (AnaeroGen Compact, Oxoid, Hants, UK, catalogue AN0010C) and transported to the laboratory for processing in an anaerobic cabinet (Whitley workstation A35).The second aliquot was used for DNA extraction and the third aliquot was processed and stored (-80°C) for biomarkers (elastase, PGP, and iron/heme).

For the cross sectional arm (Figure 2.1), patients between 6-18 years and > 18 years, were invited to give a sputum sample initially when they were stable, off antibiotics for 4 weeks. If patients were unable to expectorate the sample was obtained with hypertonic saline. For patients enrolled on the longitudinal arm (Figure 2.2), when they got an exacerbation of CF as defined by Fuchs guidelines (Flume, Mogayzel et al. 2009), a sputum, serum and urine was obtained on the day of presentation (Day 1) and following completion of antibiotics (Day 14) and a repeat set of samples was requested, when the patient was stable (off antibiotics for 1 month) this usually occurred at the next clinic visit. Control subjects were age/sex matched normal healthy volunteers (age < 6 years, age>12 years to adults) and gave induced sputum, they were recruited at each site. Spirometry was performed at each centre at least every 3 months. At the Dublin site, we followed up 83 adult patients and 58 children and young adults (0-18 year olds) over a 4 year period.

2.2 Clinical data collection and storage.

For each CF patient or control subject, clinical information (including oral exam for periodontitis, history of reflux and concomitant medications) was obtained. For CF patients, additional details on spirometry, conventional culture results, history of *P. aeruginosa*, nutritional parameters, medications, exacerbations in last year, CF complications, and genotype, were also obtained at each centre. Case report forms (Appendix 8.11) were entered into a specifically designed web-based data management system. Data was entered at each clinical centre and stored in a secure environment on the server at UNC for analysis.

2.3 Power and sample size calculations.

Before the study was commenced, power and sample size collections were made. One of the main primary endpoints overall of the study was to test for the presence of anaerobes in the CF lungs versus non CF subjects. Preliminary data from our colleagues in Belfast showed the proportion of adult CF patients with anaerobe concentrations in sputum $>10^5$ cfu/g was 27/50 and that of healthy subjects was 3/20. To detect a difference between healthy subjects and adult CF patients of this magnitude (54% vs. 15%) with 85% power, based on a 2-sided chi-squared test with $\alpha=0.05$, requires a sample size of 30 subjects per group. Further, BAL (0-6 yrs) and induced sputum (>12 yrs) may also have different characteristics. Therefore, 30 control subjects for each of the 0-6 and 12->18

year-old age groups for comparison with CF subjects between the three sites were recruited.

The second main end point was to test for an association between *P. aeruginosa* and anaerobes in CF lungs in comparsion to normal subjects.150 adult patients were recruited per age stratum (0-6 years, 6-18 years, and >18 years), for a total of 450 patients across the 3 sites. This design was done to support the assessment of clinic differences and age-group differences in the relationship between anaerobes and *P.aeruginosa*. For power calculations, we assumed that the association of *P. aeruginosa* and anaerobes would be similar to that shown in our preliminary data from our Belfast collaborators, namely that the proportion of patients with anaerobes given *P. aeruginosa* positive and negative status is 0.667 and 0.400, respectively (odds ratio of 3.0). We further assumed that 20% of the 0-6 year olds, 50% of the 6-18 year olds, and 70% of the >18 year olds would be *P.aeruginosa* positive. Based on a 2-sided, chi-squared test of association with $\alpha = 0.05$ and 150 patients in each age stratum, we calculated power of 69%, 88%, and 81% for the 0-6 yrs, >6-18 yrs, and >18 yrs strata, respectively. The power for a test of overall association based on 450 patients and stratifying by age and clinical centre via a Cochran-Mantel-Haenszel (CMH) summary chi-squared test exceeds 95%. Sufficient patients were available for recruitment, as there were 430 CF patients in Belfast, 220 in Dublin, and 480 patients at UNC, with about equal distributions between children and adults.

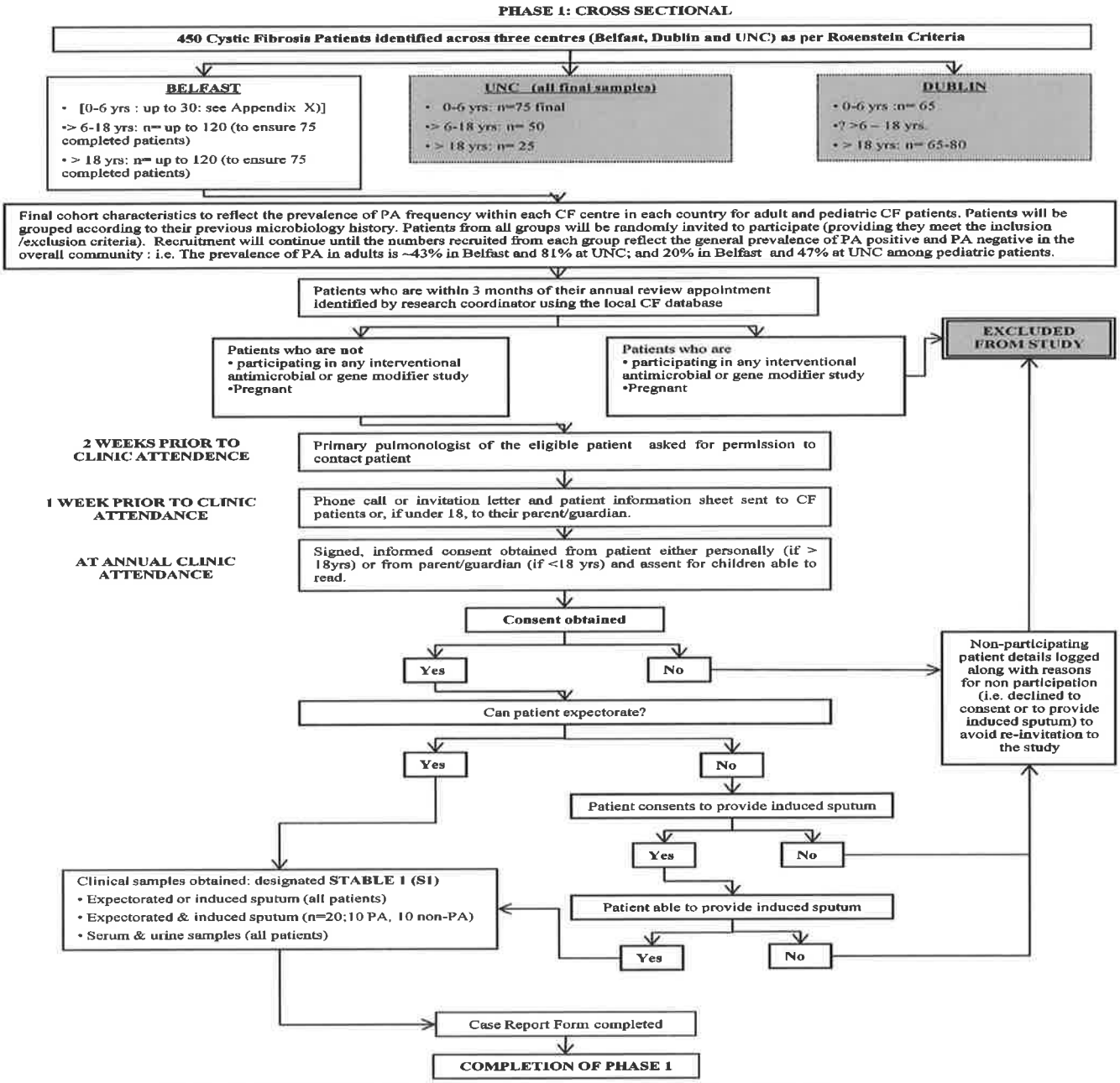


Figure 2.1: Recruitment pathway for cross sectional patients.
Adapted from protocol devised by the 3 collaboration sites

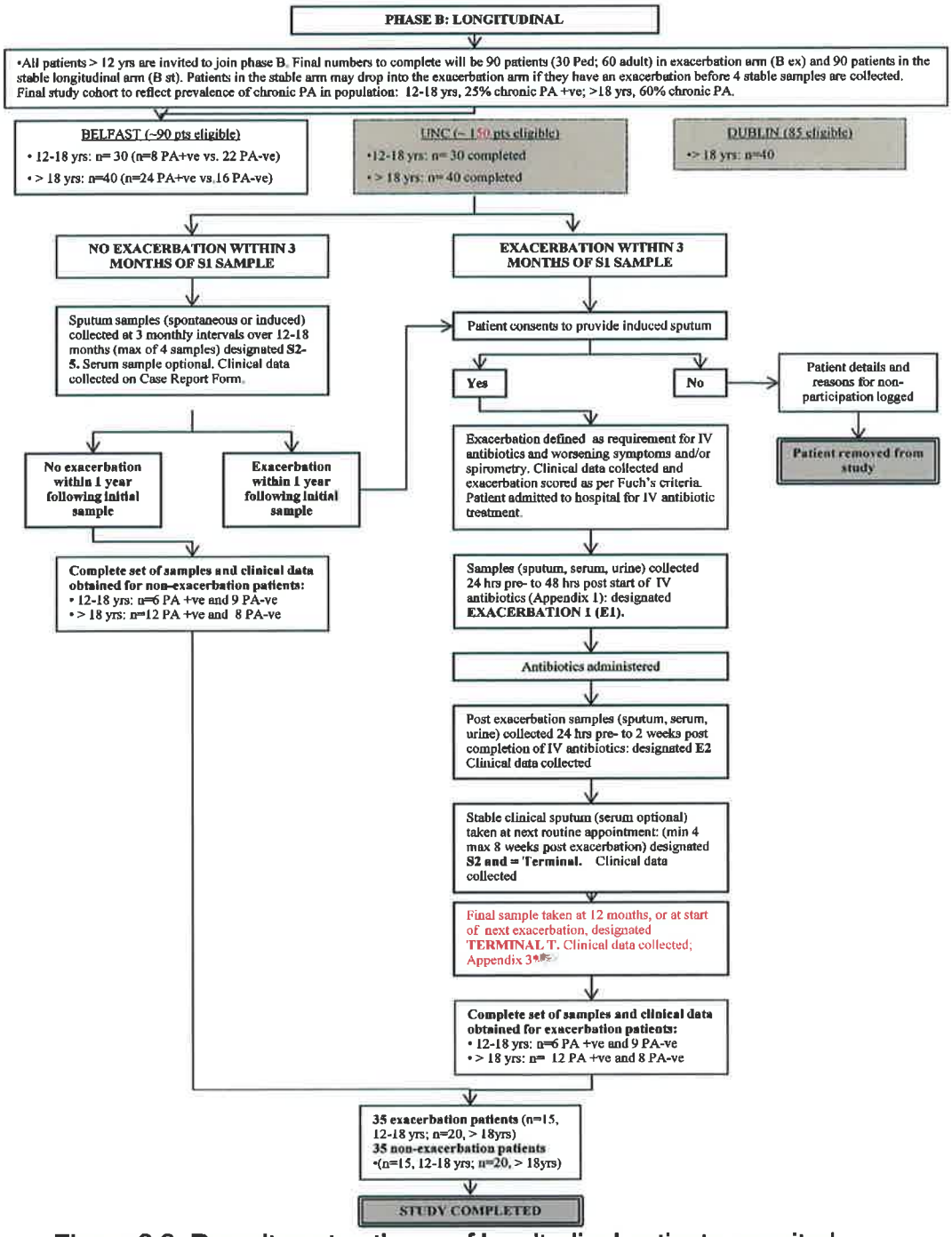


Figure 2.2: Recruitment pathway of longitudinal patients recruited. Adapted from protocol devised by 3 collaborated sites

2.4 Material suppliers

2.4.1 Antibodies and recombinant proteins

A list of primary antibodies employed in this study is found in Table 2.1. W-used for western blot analysis. Secondary antibodies and isotype controls are enumerated in Table 2.2.

Description	Catalogue number	Supplier	Source	Application	Dilution
Anti-alpha1 antitrypsin	ab7633	Abcam	Goat, polyclonal	W	1:1000
SLPI	ab46763	Abcam	Rabbit, polyclonal	W	1:1000
Elafin	sc-20637	Santa Cruz	Rabbit,polyclonal	W	1:1000
LL-37	PA-LL37100	Inovagen	Rabbit,polyclonal	W	1:1000
Lactoferrin	ab15811	Abcam	Rabbit,polyclonal	W	1:1000

Table 2.1: Primary Antibodies

Description	Catalogue number	Supplier	Source	Application	Dilution
HRP- rabbit conjugated anti-goat	Sc-2922	Santa Cruz, CA, USA	Rabbit against anti goat IgG	W	1:2500
HRP-bovine anti rabbit IgG	Sc-2370	Santa Cruz, CA, USA	Bovine against rabbit IgG	W	1:2000

Table 2.2: Secondary antibodies and isotype controls

2.4.2 Reagents

Unless stated otherwise, cell culture reagents were obtained from Gibco BRL (Karlsruhe, Germany). All other chemical reagents were purchased from Sigma-Aldrich (Dublin, Ireland) and were of highest purity available.

2.5 Cell culture

Immortalised human bronchial epithelial cells 16HBE14o$^-$ (HBE) (Cozens, Kunzelmann et al. 1994) and immortalised bronchial epithelial cells from a Δ F508/ΔF508 CF patient, CFBE41o$^-$ (CFBE)(Bruscia, Sangiuolo et al. 2002), were kindly donated by Dr. Gruenert (University of Vermont, VT,USA).

2.5.1 Maintenance of cell cultures

Cells were grown in flasks coated with fibronectin (1mg/ml), collagen (1mg/ml) (BD Biosciences, Bedford, MA, USA), and BSA (1mg/ml) in complete media (minimum essential medium (MEM)) containing 10% (v/v) heat inactivated foetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2. The media was changed every 2 to 3 days. All experiments were conducted in under10 passages. Cells were subcultured by trypsinisation. Media in flasks were removed and cells were briefly rinsed with 10ml of pre-warmed Dulbecco's PBS, calcium and magnesium free (DPBS). After carefully removing the DPBS, cells were incubated at 37°C in 4 ml of pre-warmed PET cell dissociation solution (1% (w/v) polyvinylpirrolidone, 0.02% (w/v) ethylene glycol tetraacetic acid, 8% (v/v) trypsin-EDTA solution (0.5g trypsin and 0.02% EDTA) in Hank's buffered salt solution) After 5 min, PET solution was replaced by fresh PET and cell detachment was continued for another 5 min at 37°C. Trypsinisation was stopped by addition of 10ml of complete media. Media with suspended cells was transferred to a 15ml conical tube and cells pelleted by centrifugation (250 x g for 5 min). The supernatant was discarded and cells were

resuspended in fresh complete medium and counted using a haemocytometer before being transferred to flasks or cell culture plates at the required cell density.

2.5.2 Cryogenic preservation of cell lines

For long-term storage, cells were trypsinised as described and resuspended in ice-cold freezing solution (90% (v/v) FCS, 10% (v/v) dimethyl sulfoxide (DMSO)) at 1 $x10^6$ cells/ml and transferred to cryovials. These were immediately placed at -80°C overnight and then stored in liquid nitrogen. The recovery of the frozen cells was carried out by quickly thawing cells in cryovials in a 37°C water bath before transferring them to a 10ml of pre-warmed complete media. Cells were pelleted by centrifugation at 250 x g for 5 min, and resuspended in 10ml of fresh culture media before being transferred to a $75cm^2$ tissue culture flask.

2.6 Tissue sampling

2.6.1 Sputum culture collection and initial plating of the sample

Sputum was collected from patients who were stable and antibiotic free for the previous one month. Patients were asked to give sputum sample into a sputum pot and then this sample was placed in an anaerobic bag (AnaeroGen Compact, Oxoid, Hants, UK, catalogue AN0010C) and transported to the laboratory for further processing. One sample was collected from patients on the cross sectional arm of the study. Patients who were on the longitudinal arm were asked to give a stable sample, pre and post exacerbation sample and at least another sample when they were stable.

For patients, who were unable to give a spontaneous sputum sample, they were asked to use a salbutamol inhaler immediately before been given 3mls of 3% nebulised hypertonic saline for 15 minutes under physiotherapist supervision. The sputum was collected similar to that previously described and transported in an anaerobic pouch to the laboratory. On reaching the laboratory, the sputum sample is immediately weighed.

An equal volume of sputolysin (Calbiochem 560000) was added to the sputum. The sputum and sputolysin were mixed by vortexing them together for 30 seconds. The suspended mixture was incubated at 35-37°C for 15 minutes in the anaerobic cabinet (Whitley A35 workstation) to ensure that the sputum was homogenized. Following this, the sputum was mixed for 30 seconds and 100 µl of homogenized sputum was added to 900 µl (1 in 10 dilution: 10^{-1}) quarter-strength Ringer's solution (Oxoid BR 0052G).It was mixed by repeated pupating and further serially diluting in 900 µl volumes of quarter-strength Ringer's solution using the same method, giving 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions respectively. Immediately afterwards, 100 µl of each dilution was inoculated on each of the agar plates, ABA (3 plates), KVLB (1 plate) and BCA (1 plate).ABA plates were grown both aerobically (5% CO_2/incubator) and anaerobically. The other plates were grown in the 5% CO_2 incubator.

2.6.2 Bronchoalveolar lavage (BAL) fluid sample collection and initial plating of sample

Specimens were collected on site at the time of bronchoscopy both for adults (Beaumont hospital) and children (OLHC). Bronchoscopy on children was

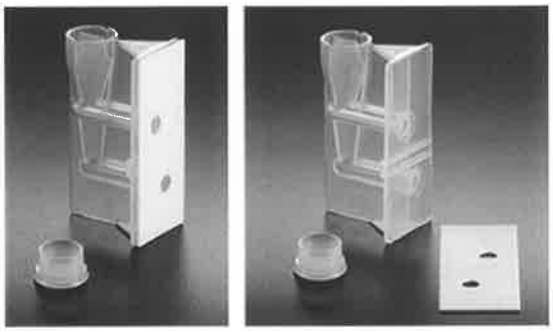
performed by a respiratory consultant via a laryngeal mask airway or endotracheal tube and the bronchoscope was directed to the lingual and right middle lobe. BAL was performed in children under general anaesthesia. BAL was performed by instilling 1ml/kg of sterile normal saline per lobe. Bronchoscopy on adults was performed under conscious sedation. The bronchoscope was directed to the area of disease both clinically and radiologically and was patient specific. BAL was performed in adults by instilling 100 mls of sterile normal saline into a specific lobe and retrieving 40mls of BAL in return. The characteristics are summarised in table 2.3. After the clinical specimen was sent to the hospital laboratory, the remaining BAL for the project was divided. 1ml was stored in a cornig tube and placed in an anaerobic pouch. The remaining volume was stored in a cornig tube and put on ice. Both samples were labelled and brought to the laboratory as soon as possible.

Processing of BAL for culture procedure was done inside the anaerobic cabinet. However, the sputolysin step was not required. 100µl of BAL was plated neat on ABA plate for aerobic culture, 100µl was plated neat on ABA plate for anaerobic culture and 100µl of BAL was added to 900µl of quarter strength Ringer's lactate solution and was plated as described before in subsection 2.6.1. For restriction fragment length polymorphism (RFLP) analysis, 1ml of BAL was required. It was centrifuged at 18,000xg for 5 minutes and then, 700µl was removed and the remaining pellet was saved. 2ml of the remaining BAL was kept for biomarkers. Initially samples were centrifuged at 3000g for 30minutes at 4°C and were placed in aliquots of 250µl of BAL for protease analysis, 250µl for

neutrophil elastase, 250µl for Iron, 250µl for PGP, 223.2µl of BAL + 27.8µl of Bestatin hydrochloride in 1:8 dilution, and 250µl of BAL + 25µl of protease inhibitor in 1: 10 dilution.

2.6.3 Cytospin protocol

20 µl of the raw BAL was added to 80µl of trypan blue. It was mixed gently together and 10µl of the cell suspension was added to the haemocytometer well. The cell count was calculated. From this cell count a cell suspension was prepared which contained 5 $x10^5$ cells/1ml using sterile PBS to dilute. Following this the Shandon cytospin apparatus was used.100µl of cell suspension was added into each side of the double funnel and spun at 1100rpm for 5 minutes. When dry, the funnel was immersed in 100% methanol for 1 minute and the sample was fixed. Slides were then stained; initially the slides were immersed in 100% May-Grunwald for 4 minutes. Then the slides were 4% Giemsa for 4 minutes. The slides were rinsed and allowed to dry. Cytoseal was placed on the slides and then a coverslip. The slide was read under a microscope and cells were counted.

A 

B

Figure 2.3: A: Cytospin funnel; B: Shandon Cytospin Centrifuge apparatus

2.6.4 Serum, plasma and urine sampling

2.6.4.1 Plasma collection

Blood samples were centrifuged in a sterile centrifuge tube at 1100-1300g for 10 minutes at room temperature. After centrifugation, the plasma was aliquoted into cryovials and stored at -80°C.

2.6.4.2 Serum collection

The serum was left to stand for 60 minutes to allow a clot to form. The serum was removed from around the clot and it was centrifuged for 20mins at 1100-1300g at room temperature. The serum samples were aliquoted into cryovials and stored at -80°C.

2.6.4.3 Urine collection

1ml of urine was frozen neat and stored in -80°C.

2.6.5 Preparation of sputum samples for analysis of inflammatory markers

The sample was split into three aliquots (microbiology (subsection 2.6.1), DNA extraction for molecular detection, biomarker analysis). 350-500mg was

removed for microbiology, 200mg for RFLP and the remainder 300mg for biomarkers. The aliquot for biomarkers was divided into three,100mg was frozen for further protease analysis. The second sample (100mg) was diluted with 8 x PBS and was centrifuged (3000 g at 4°C for 30 minutes) at 4 °C and aliquots of 250 µl were stored for neutrophil elastase, iron/haem, p-glycoprotein (PGP) and bestatin. The final sample was diluted with 1 x PBS and was centrifuged at 3000 g at 4°C for 30 minutes. An inhibitor cocktail was added at a dilution of 1:10 and stored at 80°C for the final aliquot.

2.7 Quality control and storage

2.7.1 Subculture of isolates for storage

A loop of each colony of gram negative and anaerobic bacteria were suspended in 1ml of 10% glycerol and stored in cryovials at -80°C. A loop of each colony of gram positive bacteria was suspended in cryopreservation fluid and beads and allowed to settle. Then the fluid was removed and the beads were stored at -70°C. The stored isolates of each of the bacteria grown were used in later experiments.

2.7.2 QC sampling of organisms-Kwik-stick cultures, microbiologic

Commercially made KWIK sticks, ATCC 25845- *P. melaninogenica* (*MBL0110P*), ATCC 17929- *A. odontolyticus* (MBL0939P), ATCC 10790- *V. parvula* (MBL0867P) were used in the protease experiments. The plates were incubated in the anaerobic chamber and there the sticks were unsealed and spread across the ABA plate. Following incubation, a well isolated colony from

each culture was removed using a sterile loop and this colony was transferred onto a fresh ABA plate.

2.7.3 DNA isolation from bacterial culture

DNA was extracted from pure colonies using the FastDNA kit (MP Bio, 116540400). The protocol was followed in accordance with the manufacturer's instructions. Briefly, a loopful of bacteria was resuspended in 1ml of sterile PBS. It was centrifuged at maximum speed for 5 minutes and the pellet was resuspended in 200µl of sterile PBS and 1ml of CLS-TC lysis buffer was added and the tubes were placed in the FastPrep machine, at a speed setting 6.0 for 40 seconds (MP Bio FASTPREP®-24 INSTRUMENT - 116004500). The eppendorf was centrifuged at 14000xg for 5-10 minutes to pellet the debris. 800µl of supernatant was transferred to a microcentrifuge tube and an equal volume of matrix buffer was added and the solution was mixed. It was put on the roller at room temperature for 5 minutes. Half of the suspension (400µl) was added to the spin filter in the kit and centrifuged at 14000xg for 1min. The catch tube was emptied and it was repeated for the remaining suspension.500µl of SEWS-M was added and centrifuged at 14000xg and the catch tube contents were discarded. It was centrifuged again at 14000xg with a new clean catch tube. The DNA was eluted by adding a binding matrix to the spin filter in 100µl of DES. It was incubated for 5 minutes on a heat block at 55°C. It was centrifuged at 14000g to bring the eluted DNA into a clean catch tube and the spin filter was discarded. The DNA was stored at -20°C until required for PCR.

2.8 RNA isolation and reverse transcriptase polymerase chain reaction

2.8.1 RNA Isolation

Total RNA was extracted from HBE and CFBE ($1x10^6$) cells using TRI reagent following manufacturer's instructions. This method permits the simultaneous isolation of DNA, protein and total RNA based on a single step liquid phase separation. In brief, TRI reagent (0.5-1ml) was added to cell pellets and stored at -80°C in eppendorf tubes. Samples were allowed to stand at room temperature for 5 minutes and 200µl of chloroform was added to each tube. Samples were vigorously shaken with vortexing for 15 sec and then allowed to stand for 2 minutes at room temperature. The resulting mixture was centrifuged at 12000xg for 15minutes at 4°C. This step separated the samples into three phases: the bottom red organic phase contained protein, the white thin interphase layer contained DNA and the upper colourless aqueous phase contained RNA. The aqueous phase was carefully transferred to a fresh tube and mixed with 0.5ml of isopropanol. Samples were allowed to stand for 5 minutes at room temperature. Subsequently, the RNA was pelleted by centrifugation at 12000 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed with 1ml 75% (v/v) ethanol. Samples were mixed by vortexing and allowed to stand for 5 minutes at room temperature. Total RNA was pelleted by centrifugation at 12000 x g for 15 minutes at 4°C. The supernatant was discarded and the RNA pellet was air dried for 5 minutes at room temperature. The pellet was resuspended in 30µl of DNase free water (ultrapure water treated with 0.1% (w/v) diethyl pyrocarbonate and autoclaved for 15 min to neutralise the RNAse inhibitor and avoid carboxymethylation of purine residues in RNA). The RNA

content was directly quantified using a Nanodrop 8000 spectrophotometer (Thermo Scientific, Dublin, Ireland). The isolated RNA was considered free of contaminants when the A260/280 ratio was >1.7. RNA samples were stored at -80°C.

2.8.2 cDNA synthesis

Gene expression was analysed by two step quantitative RT-PCR. In the first step RNA was reverse-transcribed to cDNA then amplified by polymerase chain reaction. Prior to cDNA synthesis, contaminating DNA was removed by the addition of 2 µl of DNase I (Qiagen, UK) to RNA samples (0.2-1µg RNA) in a total volume of 14µl, followed by 2 minute incubation at 42°C. The RNA was then reversed-transcribed using QuantiTect Reverse Transcription Kit (Qiagen) by addition of primer mix, 1µl reverse transcriptase and 4µl buffer in a total 20µl reaction volume. Samples were incubated at 42°C for 30 minutes, followed by 95°C denaturing step for 5 minutes in a PTC-200 thermocycler (MJ Research, MA, USA). Samples were employed directly for RT-PCR analysis or stored at 20°C.

2.8.3 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

cDNA (2µl) was amplified with SYBR green I Master mix (Roche, Basel, Switzerland) using the LightCycler 480 PCR system (Roche). PCR was performed according to the manufacturer's instructions using the following protocol: preincubation (95°C, 3 min); amplification, 40 cycles consisting denaturation, annealing, elongation (95°C, 10sec, 10 sec at annealing temperature indicated in Table 2.4 and 72°C for 10 sec); melting curve analysis

(95°C 5 sec, 65°C 1 min, 97°C 5 continuous acquisitions/°C); and final cooling step to 40°C, using previously described primers (NCBI protein database). The primer sequences are provided in Table 2.4. All samples were carried out in duplicate; 20µl reactions in 96-well plates and a negative control with no cDNA template were included in every run. Specificity of the amp icon products were confirmed by visual inspection of melting curves and products run on an agarose gel. The relative expression of the gene was determined using the $2^{-\Delta\Delta Ct}$ method (Schmittgen 2008) with GADPH and β actin as internal controls.

2.8.4 Agarose gel electrophoresis

PCR products (10µl) were resolved on agarose gel 1.2% (w/v) agarose, 0.01% (v/v) SyBr safe DNA gel stain (Invitrogen, Biosciences, Ireland) in bionic buffer) at 150mV for 30-40minutes using bionic buffer as running buffer.

Gene	Primers (5'→3')	Product size (bp)	Annealing T (°C)
β actin	Forward- GGA CTT CGA GCA AGA GAT GG Reverse- AGG AAG GAA GGC TGG AAG AG	138	56
FFAR 3	Forward- TTC ACC ACC ATC TAT CTC ACC G Reverse- GGA AGC GTT CAA TGC TCA CAG	58	56
FFAR 2	Forward-CCG TGC AGT ACA AGC TCT CC Reverse- GGT GCA GTG ACC AAA GGA CA	86	56
GAPDH	Forward-CAT GAG AAG TAT GAC AAC AGC CT	113	56

Reverse-AGT CCT TCC ACG ATA CCA AAG T

Table 2.3: Primer sequences and amplicon length in qRT-PCR analysis

2.8.5 PCR Primer Design

PCR primers were designed using published mRNA gene sequences for the receptors involved in SCFA, GPR 41 and 43, located using NCBI Entrez Nucleotide (NIH, Bethesda, MD) (Table 2.4). Certain criteria were considered in designing the primers. The overall purine versus pyrimidine composition was important. If possible, half or greater of the bases were G or C as this makes oligonucleotide binding to template cDNA more stable. Sequences of four or more of the same base were avoided. Also, care was taken to ensure that the two primers to be used together did not contain significant regions of complementary or inverted repeats, which would result in hairpin loop formation or primer dimer formation. All primers were designed using the Primer 3 software programme (Whitehead Institute/MIT Centre for Genome Research, Cambridge, MA) (http://www.basic.nwu.edu/biotools/Primer3) and synthesised by MWG Biotech.

2.9 Agar preparation

2.9.1 Anaerobic Basal Agar (ABA) and Chocolate blood agar containing bacitracin (BCA)

Anaerobe Basal Agar- (Oxoid, CM 0972)	46g
Defibrinated horse blood (TCS Biosciences, HB 034)	50ml
Distilled water	1000ml

Table 2.4: ABA agar

ABA was prepared by obtaining the following ingredients (Table 2.5). The anaerobic basal agar was suspended in 1L of distilled water. It was boiled in the waterbath to dissolve solution and then it was autoclaved at 121°C for 15 minutes. It was then cooled to 50°C with a thermometer checking the temperature and then blood is added. In a sterilised environment with Bunsen burner lighting or in the sterilisation hoods, 20 mls was aliquoted into each petri dish and stored at 4°C until ready to use.

This was made for the first few experiments only. ABA agar then became available to purchase (Fannin LIP, W11348). BCA used in the experiments was purchased (Fannin LIP, W11063).

2.9.2 Kanamycin-vancomycin laked blood agar (KVLB)

Brucella Agar Base (Oxoid CM 0169)	45g
Defibrinated horse blood TCS Biosciences, HB 034)	50ml
Haemin (Sigma, 51280) This is predisolved 0.1g of Haemin in 0.1M NAOH(2ml NAOH + 18ml of distilled water)	1ml
Vitamin K1 (Sigma , V3501) 0.2ml in 20ml NAOH for the stock solution then dilute 1:10	1ml
Kanamycin sulphate (Sigma, K4000)	1.5mls(Stock concentration:100mg/2ml of distilled sterile water, filter sterilise)
Vancomycin (Flynn Pharma Ltd, local hospital pharmacy)	1.5mls (Stock concentration: 10mg/2ml of sterile distilled water, filter sterilise)
Distilled water	1000ml

Table 2.5: KVLB Agar

KVLB was prepared by using components in Table 2.6. Brucella agar was added to 1 L of distilled water, and then haemin and vitamin K1 were added. It was mixed well and autoclaved at 121°C for 15mins. It was removed from the autoclave and placed in a water bath at 50°C. Added to the solution, was 1.5mls each of Kanamycin and Vancomycin stock solution (to give a final concentration of 75mg/l and 7.5mg/l respectively the horse blood was then added. It was mixed thoroughly and 20 ml aliquot was dispensed in a sterile environment into sterile Petri dishes and stored at 4°C.

2.9.3 McKay agar

Nutrient broth (BD 234000)	13.3g
Dextrose (Sigma D9434)	5g
Yeast extract (Oxoid LP 0021)	10g
K_2HPO_4 (Anala R 10349)	1g/L
$NAHCO_3$ (Sigma S-4019)	10g/L
NACL (Sigma S7653)	2g/L
KH_2PO_4 (Sigma P5379)	1g/L
$MgSO_4 * 7H_2O$ (Sigma 63138)	0.5g/L
$CaCL_2 * 2H_2$ (Sigma C7902)	0.25g/L
Tween 80 (Sigma P1754)	1ml
Crystal Violet (Sigma C 3886)	1mg
Bromcresol Purple (Sigma B5880)	60mg
Vitamin K	10µg
Haemin (Sigma H9039)	0.005g
Sulfadiazine (Sigma S8626)	16mg/ml (496mg with 31ml of 0.1M NaOH, filter sterilised)
NAOH	
Colistin sulphate (Sigma C4461)	10mg is mixed with 10ml of distilled water and then filter sterilised
Oxolinic acid (Sigma O0877)	5mg is dissolved with 10ml of 0.1M NaOH, which is then filter sterilised in the fume hood
Bacto agar (BD 214530)	15g/L
L-Arginine (Sigma A5006)	20ml (625mg in 20ml of distilled water, filter sterilised)

Table 2.6: McKay Agar

A one litre flask of distilled water was obtained and nutrient broth, dextrose, yeast abstract, tryptone and K_2HPO_4 were added (Table 2.7) 40 mls from the stock salt solution was added to the mixture(Salt solution- $NaHCO_3$, NaCL, K_2HPO_4, KH_2PO_4, $MgSO_4*7H_2O$, $CaCL_2*2H_2O$). Then tween, crystal violet, bromcresol purple, vitamin K, haemin and bacto agar were added.

Before adding the antibiotics, the pH was adjusted to 7.2 and then sterilised in the autoclave 121°C for 15 minutes. In a sterilised hood, 20ml L-Arginine (2.5% w/vol) was added and filter sterilised antibiotic stocks of the following, sulfadiazine (16mg/ml in 0.1M NaOH), colistin sulphate (1mg/ml in distilled water) and oxolinic acid (0.5mg/ml in 0.1M NAOH) were added to the final concentrations of 500µg/ml, 10µg/ml and 5µg/ml respectively.

2.9.4 LB Broth

10 tablets of LB broth (Sigma L 7275-500 tab) were made in 500mls of distilled water in a glass jar. The broth is then autoclaved, at 121°C for 15 minutes, and in a laminar flow hood, 10 mls of broth was aliquoted as required into corning tubes and stored until ready to use at 4°C.

2.9.5 BAM

Solution 1	Solution 2	Solution 3	Solution 4
Tryptone Soya Broth Fluka: T9410 5g	$NaHCO_3$ (Sigma S-4019) 4g	Cysteine Hydrochloride (Sigma C1276) 0.5g	Haemin chloride type 1 bovine (Sigma H9039) 50mg

Proteose Peptone (Sigma 82450) 5g	Distilled water filter sterilised 100ml	Distilled water filter sterilised 10ml	K_2HPO_4 (Anala R 10349) 1.74g
Yeast extract - (Oxoid LP 0021) 2.5g			NAOH 0.4g
Sodium Chloride (Sigma S7653) 2.5g			Distilled water 99ml
Distilled water			Menadione stock solution- Menadione Vitamin K3 (Sigma 47775)- 100mg Ethanol 20ml
pH adjusted to 7.4 and autoclaved			

Table 2.7: BAM broth

BAM media was prepared aseptically. All 4 solutions were added and it was microwaved at full power in a glass conical flask for 3 mines to displace dissolved oxygen, after cooling, media was transferred into an anaerobic cabinet. 100mls of BAM was from the following solutions: Solution 1- 97mls of stock, Solution 2- 1ml of stock, Solution 3-1ml of stock and Solution 4-1ml of stock solution.

2.9.6 Gram staining

A drop of water was placed on a slide that was sterilised (it was placed first over Bunsen burner heat); the bacteria colony was emulsified and dried at the Bunsen burner. When it was dry, it was immersed into the crystal violet

(Fluka 94448) for 1 minute, washed off with decolouriser solution (Fluka 75482), and then iodine solution was added for 1 minute (Fluka 90107) and washed off with 95% ethanol for 5 seconds. The slide was flooded with distilled water and the safranin was left on for 4 minutes (Fluka 94635). Then slide was allowed to dry and it was viewed under the microscope with immersion oil (Sigma 56882) at power 40.

2.10 Immunoblotting

2.10.1 SDS-polyacrylamide gel electrophoresis

Proteins were separated by SDS- page according to Laemmli's method (Laemmli 1970). Samples were stored at -80°C until ready for use, they then are thawed. Samples (10µl) were denatured by adding sample loading buffer (2µl) and heated for 3 minutes at 99°C. Gradient gels were required, NuPage Bis-Tris 4-12% acrylamide precast gels (Invitrogen). Briefly, precast gels were rinsed in deionised water and mounted in a Xcell SureLock Mini-cell system (Invitrogen). SeeBlue Plus2 Prestained molecular mass markers (5µl; Invitrogen) were loaded on each gel for determination of molecular weight. The electrophoresis chamber was filled with NuPage running buffer in non reducing conditions (50ml Nu Page 20X (Invitrogen), 950ml deionised water) and samples were loaded. Electrophoresis was carried out for 50 min at 200V.

2.10.2 Western Blot analysis

Following electrophoresis, the resolving gel was equilibrated for 1 minute in NuPage Tris-Glycine transfer buffer (40ml NuPage 25x (Invitrogen),760 ml deionised water, 200ml methanol) and placed on top of a 0.2µm pore size nitrocellulose membrane and sandwiched between Whatmann chromatography paper (Whatmann International Ltd, Maidstone, England) pre-soaked in transfer buffer.

Proteins were transferred onto membranes at 150mA for 60 minutes using a semidry blotting apparatus. Following transfer, membranes were blocked with

5% (w/v) non-fat powdered milk in PBS containing 0.1% (v/v) Tween-20 (PBST) for 1 hour at room temperature. PBST contained 3% (w/v) BSA was used as a blocking buffer. For immunological detection of the desired proteins, blots were incubated overnight at 4°C in blocking buffer containing antibody against AAT. Subsequently, nitrocellulose membranes were washed for 30 minutes PBST buffer (x2), probed with corresponding HRP-conjugated secondary antibody in PBST for 1 h and then washed again. Blots were developed with Immobilon western chemiluminescent HRP substrate (Millipore, MA, and USA) and visualised on the Syngene G: Box chemi XL gel documentation system (Synoptics, Cambridge, UK). Protein band size was estimated by comparison with molecular weight markers.

2.10.3 To determine which proteases are involved in AAT degradation

The following inhibitors were used in these experiments at concentrations that were determined in previous publications to be present (Guyot, Zani et al. 2005). Aprotinin (1M solution ,Sigma Aldrich- 10820), α 1 antichymotrypsin (ACT) (Sigma Aldrich -A9285, 1μM solution), phenylmethanesulfonyflonade (PMSF) (Sigma Aldrich- P7626, 10mM solution),pefabloc (Sigma Aldrich, 10mM solution), pepstatin A (Sigma Aldrich, 0.4mM solution) and ethylenediamine-tetraacetic acid (EDTA) (Sigma Aldrich,13mM solution). E-64 (Merek Biosciences, 0.4mM solution) and human neutrophil elastase (NE) (EC 3.4.21.37) were purchased from Elastin Products Company, Inc. (Owensville, MO, USA). Purified alpha-1-antitrypsin (AAT) was from Athens Research

(Athens, GA, USA). Complete protease inhibitor tablets were obtained from Roche.

Equal amounts of bacterial supernatants from *P. melaninogenica* and inhibitor concentrations were added together. AAT (protein concentration 3µg/dl) was subsequently added and the inhibition was determined by western blot analysis as described in 2.10.2.

2.11 Zymogram analysis

Zymogram analysis was used for detecting and characterising metalloproteinases, collagenases and other proteases that can use casein or gelatine as a substrate. Protease samples are initially prepared in SDS buffer under non-reducing conditions and without heating. Samples were prepared by using 10µl of *P. melaninogenica* supernatant incubated in LB broth and BAM media on various day 3-7 and BAM and LB broth media acting as a control. Each sample was added to 2µl of SDS non-reducing buffer. Novex Zymogram gels (Invitrogen) were used and the protocol was followed according to the manufacturer's instructions. Briefly, the running buffer was prepared by adding 1X Tris-Glycine SDS running buffer to 100ml of 10X Novex Tris Glycine SDS running buffer to 900ml of deionised water. The precast gels were rinsed in deionised water and mounted in a Xcell SureLock Mini-cell system (Invitrogen), the sample was then loaded and the chambers were filled with running buffer. Electrophoresis was carried out for 90 minutes at 125V constant. After the electrophoresis the enzyme was renatured and developed to detect protease

activity. Novex Zymogram renaturing buffer10x was diluted 1:9 dilution with deioninsed water giving a total volume of 100ml. The gel was removed from the cassette and incubated with the 1X renaturing buffer for 30 minutes at room temperature on a rotator. Novex Zymogram developing buffer10x was diluted 1:9 dilution with deioninsed water giving a total volume of 100ml. The renaturing buffer was decanted and the developing buffer 1X was allowed to equilibrate with the gel for 30 minutes with gentle agitation. Then the buffer was discarded and a fresh 1X developing buffer was added and incubated at 37°C overnight. Then the gel was stained with Coomassie blue stain for an hour followed by destaining buffer for 1-2 hours (Table 2.9). Blots were visualised on the Syngene G: Box chemi XL gel documentation system (Synoptics, Cambridge, UK).

Coomassie Blue Stain-500mls	Destain-Coomassie- 500mls
500ml (10%) acetic acid	50ml (10%) acetic acid
225ml (45%) methanol	125ml (25%) methanol
225ml (45%) deionised water	325ml (65%) distilled water
1g (0.2% w/v) Coomassie blue	Tissue to soak stain

Table 2.9: Coomassie Stain and Destainer

2.12 NE Activity Assay

NE activity in stock preparations was determined before each experiment using the NE-specific substrate N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (Sigma). Liberation of p-nitroaniline was measured at 405nm after a 5 minute time period. NE activity was quantified by comparison with an NE standard of known activity.

2.13 Enzyme linked immunosorbent assay (ELISA)

The levels of a specific protein can be easily measured using ELISA. This method is based on the formation of an antigen-antibody reaction that results in a highly specific and sensitive measurement of analytes in large numbers of biological samples (Greene 2010).

2.13.1 Determination of cytokine levels

IL-8 protein concentration in cell supernatants was determined by sandwich ELISA (R & D Systems) according to manufacturer's instructions. When confluent, 1 x 10^5 CFBE cells and HBE cells were grown overnight in media for 24 hours in a 24 well plate, subsequently they were serum starved for

another 24 hours. 0.15 M solution of sodium acetate, sodium propionate and sodium butyrate was made up in PBS and pH was checked to be between 7-7.5.

On Day 3, an experiment was set up, n=3. Each treatment was done in triplicate and done to both HBE and CFBE cells simultaneously. Equal volumes of solutions were used. Control sample, 33µl PBS, 967µl SFM, 1/2000 dilution was made for the positive control PMA, 33µL of this was added to 967µl of SFM, each of the above fatty acids,33 µl was added with 967µl of SFM. These were incubated over 24 hours and then stored at -80°C until ready to do an ELISA.

Briefly, high binding Immulon 2HB flat bottom polystyrene microtiter plates (Immunolon; Thermo Scientific, Rochester, NY, USA) were coated with 100µl of the IL-8 capture antibody (MAB 208) at 2µg/ml in Voller's buffer (25mM Na_2CO_3 and 15mM $NaHCO_3$ in deionised water, pH 9.6) overnight at 4°C. Following this, wells were rinsed with wash buffer (1% (v/v) Tween-20 in PBS), blocked with 200µl of 1% (w/v) BSA dissolved in wash buffer and incubated for 1hour at room temperature. After washing wells (x3), 100µl of samples or IL-8 standard diluted in PBS (0-2000 pg/ml) were pipette in wells. 100µl of SFM and 100µl of supernatants from cells for IL-8 quantification by ELISA to ensure values were within normal range of detection. Following 2 hour incubation at room temperature, the wells were washed again and incubated for a further 1h with 100µl of IL-8 detection antibody (BAF 208) diluted to 0.1µg/ml in washing buffer.

The wells were then washed and incubated with 100µl of streptavidin-HRP (Biolegend, 405210) at 250µg/ml in washing buffer. Following 1hour incubation at room temperature, wells were washed and 100µl of 2, 2'-azino-bis (3-

ethylbenzothiazoline-6-sulphonic acid), (ABTS, Invitrogen) was added for 30 min. All measurements were conducted in duplicate and recorded using a Wallac 1420 Victor2 multilabel counter set at 405nm.

2.14 Sensolyte Assay

A sensoLyte^{TM} red protease assay kit (Ana Spec, 71140) was used to detect generic protease activities (trypsin, chymotrypsin, thermolysin, proteinase K, protease XIV and elastase) using casein that is heavily labelled with 5 (6) TAMRA, a pH insensitive red flurophone that yields a red fluorescence, which can be continuously monitored at excitation/emission= 546nm/575nm. The increase in fluorescence intensity is directly proportional to protease activity. Protocol for measuring protease activity was followed according to manufacturer's instructions. *P. melaninogenica* was grown in various media from day 3 to day 7 and the bacterial supernatants were extracted daily. Once obtained they were centrifuged at 6200xg for 5 minutes, a pellet formed and the supernatant was labelled in an eppendorf and stored at -80°C. Prior to using the assay, the protein was concentrated further using centrifugal filters (Amicon Ultra-0.5) giving a final volume of 20µl. 50µl of the protease substrate solution the kit was added to each of the components of the assay giving a final volume of 100µl in each well of a 96 well plate (Table 2.10). The assay was followed according to the manufacturer's guidelines and the fluorescence signal was measured at Ex/Em=546nm/575nm.

Negative control	Positive control	Test sample
Deionised water 50µl	Trypsin diluents 50µl	Protease containing
		Sample 50µl

Table 2.9: Components of Sensolyte assay

2.15 Short chain fatty acid extraction (Zhao, Liu et al. 2007)

2.15.1 Equipment

Chromatographic analysis was carried out using a Varian CP3800 GC equipped with a flame ionization detector and a manual liquid sampler. A column (Varian WCOT Fused Silica 100m x 0.25mm ID Code: CP7420) was used. Data handling was carried out with Dell Optiplex GX240 using Varian STAR software. The polyprolene hollow fibre tube (50µm wall thickness, 280µm inner diameter, 0.1µm pore size, model 50/280 Accurel PP) was obtained from Membrana GmbH, Wuppertal, Germany. BD Micro-Fine syringes (with needle of 0.30mm outer diameter, 8cm length, 0.5ml holding volume and prepared for U-100 insulin injection) were obtained from a local pharmacy and used to fill the acceptor into the lumen of the hollow fibre for extraction and to flush out the acceptor from the lumen of the hollow fibre into a 100µl pulled point conical glass vial (Sigma-Aldrich, St. Louis, MO, USA).

2.15.2 Materials

High purity SCFAs were used to prepare standard solutions. High purity solutions of acetone, valeric acid, proprionic acid, butyric acid, i-butyric acid, i-

valeric acid and 2-ethylbutyric acid as an internal standard were purchased from Sigma-Aldrich (Chemie GmbH, Steinheim, Germany). An aqueous stock standard solution was prepared for each acid with a concentration of 25mM for ACE,1mM for PRO, BUT and IBUT, 3mM for VAL and 0.5mM for IVAL. A working standard solution was then obtained and an internal standard stock solution containing 2-ethylbutyric acid with 12% formic acid was also prepared. Tri-n-octlyphosphine oxide (TOPO) and dihexyl-ether (DHE) were obtained from Sigma Aldrich. The organic membrane liquid layer was prepared by dissolving appropriate amounts of TOPO in DHE. Analytical grade-HCL and formic acid were used. The donor was acidified using 12mM HCL and 12% (v/v) formic acid to clean the GC column. Analytical grade NaOH was used as a basic acceptor.

2.15.3 Sample preparation

KWIK sticks (Microbiologics, Minessota, USA) of *Streptococcus sanguinis* (ATCC ID: 10556), *Fusobacterium nucleatum* (ATCC ID: 25586), *Actinomyces odontolyticus* (ATCC ID: 17929), *Prevotella melaninogenica* (ATCC ID: 25845), *Veillonella parvula* (ATCC ID: 10790) and *Pseudomonas aeruginosa* (ATCC ID: 15962) or bacterial isolates from patients (from the larger study "Anaerobic bacteria in CF sputum") were inoculated in basal anaerobic medium (BAM) and grown under anaerobic conditions at 37°C. Optimal incubation time was determined in a preliminary experiment where anaerobes were grown for 7 days and the day with the highest response in SCFAs production was chosen for further work. After appropriate incubation time under strict anaerobic conditions

the bacterial supernatants were prepared by centrifugation at 6200g for 5minutes and stored at -80°C.

To prepare the donor solution for SCFAs extraction an appropriate volume of bacterial supernatant was diluted to 1.5ml using water and acidified to pH 2 by 2M HCl (see Table 2.11). Each experiment was performed in quadruplicates.

Bacteria	Incubation time (d)	Volume of bacterial supernatant (µl)	2M HCl (µl)	ddH20 (µl)
Actinomyces odontolyticus	2	50	40	1410
Streptococcus sanguinis	2	50	40	1410
Veillonella parvula	6	17	40	1443
Prevotella melaninogenica	4	100	40	1360
Fusobacterium nucleatum	2	17	40	1443
Pseudomonas aeruginosa	1	100	40	1360

Table 2.10: Preparation and incubation time of donor solution

2.15.4 Extraction procedure

The extraction procedure was carried out as described previously (Zhao, Liu et al. 2007). The lumen of a piece of hollow fibre (~16 cm) was flushed and filled with acceptor solution (0.3M NaOH) by using a BD micro-fine syringe. The fibre was then dipped into the membrane liquid (10% TOPO (m/v) in DHE) for a few seconds, to form the organic liquid membrane. Afterwards the fibre was flushed and filled again with the acceptor solution and sealed at both ends using tin foil. To remove the surplus membrane liquid the fibre was washed in water for 1 minute and then transferred to a 2.0 ml glass vial (Sigma-Aldrich, St. Louis, MO, USA) containing 1.5ml of donor solution. The latter was prepared

beforehand by dilution of standard mixture or bacterial supernatants with water and acidifying the solution to pH 2 using 2M HCl (see above). The vial was then put on the roller mixer and after 16 hours one of the sealed ends was cut and the acceptor solution with the extracted analytes was harvested in a dry 1.1ml conical glass vial using the BD micro-fine syringe. 4µl of harvested analyte was transferred into a new 1.1ml conical glass vial to which 5µl of 40mM 2-ethylbutyric acid was added. Samples prepared in this way were then analyzed using gas chromatography.

2.15.5 Gas chromatographic analysis

Nitrogen was supplied as a carrier gas at a flow rate of 1 ml/min. The initial oven temperature was 100°C and was kept there for 0.5min and raised to 160°C by 4°C/min and held there for 1 min, it further increased to 180°C by 3°C/min and held there for 1 min, then further increased to 200°C by 4°C/min and held there for 2 min. The temperature of the FID and the injection port were 240 and 200°C, respectively. The flow rates of hydrogen and air were 30 and 300ml/min. The injected sample volume for GC analysis was 2µl and the running time for each analysis was 31min. 12% (v/v) formic acid was used to clean the GC column.

2.16 Short Chain Fatty acid identification, quantification and expression

2.16.1 Identification and quantification of SCFAs

Identification of SCFAs in the analysed samples was done by comparison of retention times of chromatogram peaks in the sample with the retention times

of peaks obtained by running the SCFAs standards on the GC apparatus (Fig 2.4).

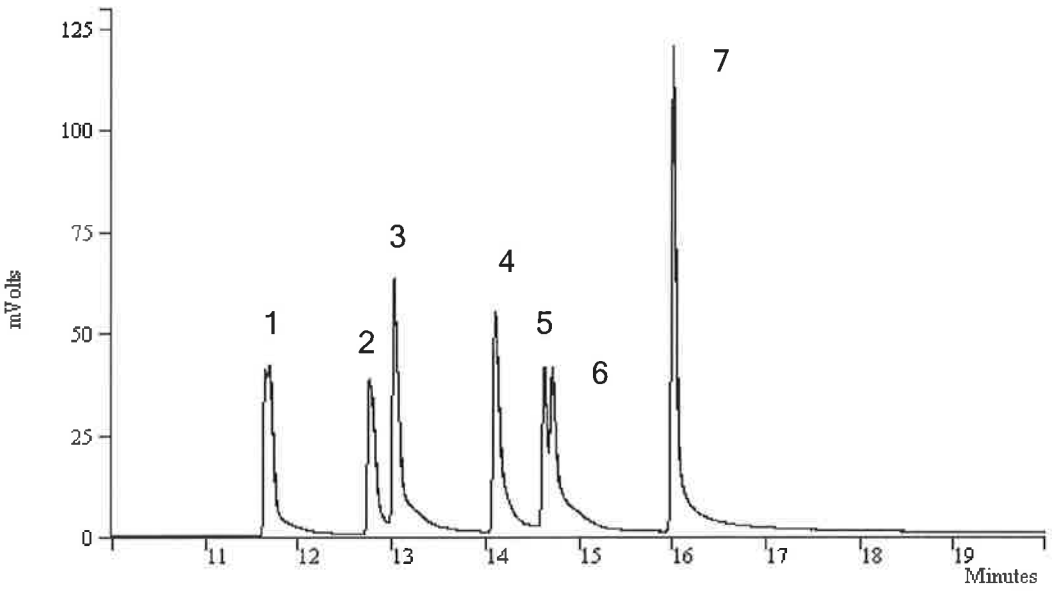


Figure 2.4: A representative gas chromatogram of SCFAs standard mixture. Peak identification: 1, acetic acid; 2, propionic acid; 3, *i*-butyric acid; 4, butyric acid; 5, 2-methylbutyric acid; 6, *i*-valeric acid; 7, 2-ethylbutyric acid (internal standard).

To aid the quantification of the SCFAs in the samples analysed 2-ethylbutyric acid was used as an internal standard to correct for variations in injection volume and other errors that might occur during the analysis. Also, a standard mixture of 8 SCFAs, including 2-ethylbutyric acid, was run on the GC apparatus and the values obtained (Table 2.12) were used to calculate the levels of individual SCFAs in the analysed samples. Using the **Equation A** the corrected peak areas were determined for each SCFAs identified in the sample.

Equation A

$$A_{Cx} = A_x \times (A_{2EBAStandard} / A_{2EBA\ Sample}) \times ([C_{2EBA\ Sample}] / [C_{2EBA\ Standard}])$$

A_{Cx} denotes the corrected peak area for the SCFA in question. A_x denotes the area of the peak of SCFAs in the chromatogram of the analysed sample. $A_{2EBAStandard}$ and $A_{2EBA\ Sample}$ denote the peak areas of the internal standard in the chromatograms of the standard mixture and the sample, respectively. $[C_{2EBA\ Sample}]$ and $[C_{2EBA\ Standard}]$ denote the concentration of 2-methylbutyric acid in the sample and standard mixture, respectively.

The concentration of SCFA ([SCFA]) in analysed samples can then be calculated using the corrected peak areas (A_{Cx}) using the **Equation B.**

Equation B

$$[SCFA] = A_{Cx} \times [SCFA_{Standard}] / A_{SCFA\ Standard}$$

$[SCFA_{Standard}]$ and $A_{SCFA\ Standard}$ denote the concentration and the peak area of the SCFA in question in the chromatogram of the standard mixture.

SCFA	Concentration (mM)	Retention time (min)	Peak area
Acetic acid	250	11.582	345627
Propionic acid	100	12.698	251560
i-Butyric acid	94.8	12.969	419076
Butyric acid	100	14.041	346384
2-Methylbutyric acid	50	14.573	166591
i-Valeric acid	50	14.659	288062
Valeric acid	22.55	15.961	39874
2-Ethylbutyric acid	23.53	16.126	103649

Table 2.12: Concentration, retention time and peak areas of the relevant SCFA

2.16.2 Linearity and enrichment factor

Linearity of the method was determined by injecting different dilutions of the standard mixture on GC apparatus. The linear range of the method was determined from graphs obtained by plotting the corrected peak areas of the individual SCFAs against the concentrations of SCFAs in the donor solution. The enrichment factor was simultaneously determined by dividing the obtained values of SCFA concentrations that correspond to the concentration of analyte in the acceptor solution after the extraction procedure with the concentrations of SCFAs in the donor solution prior the extraction procedure.

2.16.3 Laser Scanning Cytometry

GPR 41 and 43 expressions on airway epithelial cells were measured by laser scanning cytometry (LSC). Cells (1×10^5) were seeded in eight-well LabTek chamber glass slides (Nalgene Nunc, Roskilde, Denmark) and incubated overnight in complete medium. The medium was then aspirated and cells were fixed with methanol for 10 min at room temperature. Slides were washed with PBS (x 3) and blocked with 2% (w/v) BSA in PBS for 15 min at room temperature. After washing with PBS, cells were incubated at 4°C in the dark with primary Ab (1:20 in PBS) for 30 min. Then slides were washed and incubated with FITC-labelled secondary Ab (1:40 in PBS) for 30 minutes at 4° C in the dark. Control chambers were probed with secondary Ab only. Slides were washed with PBS; cells were then taken and put in permeabilizing buffer (0.1 % [w/v] sodium citrate, 0.1 % [w/v] Triton X-100 in PBS) and 0.1µg/ml solution of propidium iodide (Molecular Probes, Leiden, The Nederlands) in PBS. Slides were washed with PBS and GPR protein expression was quantified on a CompuCyte laser-

scanning cytometer (CompuCyte, Westwood, MA). Cell nuclei were identified by propidium iodide fluorescence (588 +/- 10 nm) and GPR surface expression were detected by FITC fluorescence (530 +/- 20 nm) and quantified. At least 3 x 10^3 cells were counted in triplicate in each well.

2.16.4 Whole cell lysates-determining protein expression

Whole cell lysates were prepared by seeding 1 × 10^6 CFBE or HBE cells in flasks coated with fibronectin (1mg/ml), collagen (2.9mg/ml) and BSA (1mg/ml). When cell reached confluence they were washed with PBS and lysed with 0.5ml radio immunoprecipitation assay buffer (RIPA buffer; 150 mM NaCl, 50mM Tris-HCL, pH 8, 0.5% [w/v] sodium deoxycholate, 1% Triton X-100 [v/v], 0.1% [w/v] SDS) supplemented with protease inhibitors Complete Mini tablets (Roche, Basel, Switzerland). After 20min on ice the lysates were clarified by centrifugation for 20min at 12000rpm and 4°C.

Cell membrane fractions were obtained by plating 1 × 10^6 CFBE or HBE cells in 10cm dishes coated with fibronectin (1mg/ml), collagen (2.9mg/ml) and BSA (1mg/ml). When cell reached confluence they were washed with PBS and scrapped off in 1ml Lamberth's break buffer (10mM KCL, 3mM NaCl, 4mM $MgCL_2$, 10mM PIPES, pH 7) supplemented with protease inhibitors Complete Mini tablets. Cell lysates were sonicated on ice for 5 s at 20W output with 1min resting intervals (3X). The lysates were clarified by centrifugation at 240g at 4°C for 10 min. Clarified lysates were ultracentrifuged at 100,000g for 1h at 4°C to obtain a crude cell membrane fraction. The crude membrane pellet was resuspended in 1ml of RIPA buffer containing protease inhibitors and homogenised using a 1-ml syringe and a microlance 3, 21 G 1.5 inch needle (BD,

Oxford, U.K.). The resuspended crude membrane lysate was clarified by centrifugation.

The protein concentration in whole cell lysates and crude membrane fraction was determined using BCA protein assay reagent from Thermo Fisher Scientific Inc. (Rockford, IL, USA).

Proteins were separated by SDS-PAGE on 12.5% (w/v) polyacrylamide gels and transferred to a PVDF membrane. After blocking step with 5% (w/v) nonfat powdered milk in PBS containing 0.1% (v/v) Tween 20 (PBST) for 1 h at room temperature the blots were incubated overnight at 4˚C in blocking buffer containing antibodies against GPR41 (sc-98332; 1:200), GPR43 (sc-28420; 1:200) and GAPDH (sc-255778; 1:1000) or E-cadherin (sc-21791; 1:500) as a loading control marker where required. Subsequently, the membranes were washed for 30 min in PBST buffer, probed with corresponding HRP-conjugated secondary Ab (1:1000) in PBST for 1 h. After washing, blots were developed with Immobilon western chemiluminescent HRP substrate (Millipore, MA) and visualized on the Syngene G: Box chemi XL gel documentation system (Synoptics, Cambridge, UK). Protein band size was determined by loading SeeBlue plus2 prestained molecular mass marker (Invitrogen, Bioscience, Ireland).

2.16.5 Bronchial brushing sample collection

6 individuals were recruited for this study as part of the larger clinical anaerobic bacteria in CF sputum study. 6 patients (four male and 2 female) had CF confirmed by sweat testing or genotyping. The patient characteristics were

summarised as follows; n=6; age 24.3+/- 4y; % forced expiratory volume in 1 sec 54.6+/- 36.1. All patients (controls and CF) were undergoing diagnostic and or therapeutic flexible bronchoscopy for clinical reasons and were also enrolled in the study. Full informed consent was obtained before the procedure according to a protocol approved by Beaumont Hospital Ethics Committee. Before withdrawal of the bronchoscope, an area 2cm distal to the carina on the medial aspect in either the right or left main bronchus was selected and washed twice with 10ml sterile 0.9% [w/v] NaCL. Subsequently, a sterile 10 x 1.2mm bronchial brush (Olympus Medical Systems, Tokyo, Japan) was inserted through the port on the bronchoscope, and the chosen area was sampled with a brush by gently scraping the selected area. The brush was withdrawn and immediately placed into complete media. Brushes were gently agitated to dislodge cells into medium, which was centrifuged at 200 x g for 5 min and cell pellets resuspended in 0.5ml TRI reagent for RNA extraction and quantitative real-time PCR (QRT-PCR).

2.16.6 Agarose gel electrophoresis

Initially 2% of agarose gel was prepared by adding 1g to 50mls of 10 x Bionic Buffer (Sigma Aldrich, Germany). It was heated in the microwave at medium heat for 30 seconds until dissolved and 5µl of SyBr Safe was added. The gel was poured when the agarose had cooled to about 55° C. The comb was inserted and allowed to cool further for another 20 minutes. The comb was removed and run in 0.5X of bionic buffer. Each sample, 10µl was loaded together with 2µl of DNA loading agent. Electrophoresis was for 30 minutes at 50mV and afterwards the gel was visualised on the chemiluminescence.

2.17 Statistical Analysis

Data were analysed with GraphPad Prism 3.0 software package (GraphPad Software, San Diego, CA). Results are expressed as mean +/- standard error of the mean (SEM) and were compared by Students one-tailed unpaired t test. Differences were considered significant when the P value was < 0.05. In Chapter 5, Figures 5.1-5.7 use r^2. The relationship between the acid and peak response was calculated using linear regression (Table 5.1), using GraphPad Prism 3.0 software. The goodness of fit was expressed at r^2.

Results: Recruitment

Chapter 3

3.1 Introduction

The potential role for anaerobic species in the pathogenesis of CF airway disease has gained interest with the advancement of molecular microbiology, however, the significance is uncertain(Tunney, Klem et al. 2011).It has previously been shown that there is higher diversity of strict anaerobes with those who are younger and have a better lung function(Cox, Allgaier et al. 2010). In our study, we wished to understand the complexes of bacterial diversity present in both children and adults with CF and if this changed with treatment of antibiotics. The presence of anaerobes in CF sputum was described by Jewes and colleagues (Jewes and Spencer 1990), where 23% of anaerobes from CF patients were found. Their study did not address the pathogenicity of anaerobes, but observed that anaerobes from patients attending clinic are not infective, as those admitted for acute exacerbations. This led them to suggest that perhaps the anaerobes have a more colonising role than a pathogenic role. Almost twenty years on from their study, the pathogenicity versus colonisation role of anaerobes in CF remains largely unanswered. Tunney and colleagues (Tunney, Field et al. 2008) found that patients that were *P. aeruginosa* positive were much more likely to have anaerobic bacteria in their sputum and they found that 64% of sputum samples from adult CF patients had anaerobes present leading them to suggest that if these anaerobes are causing infection and inflammation, more targeted therapies should be developed in order to treat them and this may improve management.

The detection of these bacterial species in respiratory samples does not necessarily mean that they have a clinically important role in infection, but the

clinical relevance has yet to be determined. In our experiments, we tried to initially find out what diverse microbiology was present in our patients and if it was different in paediatric versus adult population groups and how the microbiology differed in *P. aeruginosa* positive patients.

3.1.1 Paediatric Recruitment

Children with cystic fibrosis and children without CF and excluding frequent lower respiratory infections, bronchiectasis, immunodeficiencies and primary ciliary dyskinesia were asked to participate in the study at outpatient clinics and on the clinical wards. Of those, that were initially asked to participate, patients were further screened and only those over 12 years of age that were productive of sputum were accepted. Younger children typically gave much smaller samples and often had a throat swab instead, which was deemed as insufficient in order to detect anaerobes in the study. 58 CF children were recruited for the study between three sites, Our Lady's hospital for Sick Children, Crumlin, Children's' University hospital, Temple Street and Beaumont hospital, Dublin from 2010 until 2013. The recruitment in the Republic of Ireland is part of a wider project involving University of North Carolina and Queen's University Belfast with the aim of recruiting 450 patients between the three sites. The cross-sectional approach stratified CF patients into 3 age groups: 0-6 years, >6-18 years and >18 years. In the Dublin cohort, BAL was obtained from children under 6 years of age from Crumlin and sputum was obtained in children over 12 years of age from Beaumont hospital, Crumlin and Temple Street hospitals. Prior ethics approval was sought and granted at all sites. BAL samples were obtained from children as part of the annual surveillance screen that under 6 year olds undergo

at the hospital and samples were sent primarily for clinical microbiology, with excess sample being donated to the study.

Sputum samples were obtained from children over 12 years of age, after obtaining prior consent. A minimum amount of sputum was required for the study and this was only taken after the sputum was sent for clinical microbiology and if there was an excess amount of sputum available. Longitudinal samples were taken from children who consented from this group, however there was difficulty in obtaining post exacerbation samples after the children had been treated for 14 days of antibiotics and this was primarily due to the fact that they were now non-productive. Control samples were also taken for the study. Consent was obtained from guardians to enrol patients in the study group. Patients who were undergoing a bronchoscopy for other reasons other than CF were asked to enrol in the study. However, patients who had a known history of bronchiectasis, primary ciliary dyskinesia and frequent chest infections were excluded from the study. For BAL samples, patients were only enrolled if they were off antibiotics for a minimum of 4 weeks and so were classified as having "stable samples".

For each CF patient or control subject, clinical information (including oral exam for periodontitis, history of reflux and concomitant medications) was obtained. For CF patients, additional details on spirometry, conventional culture results, history of *P. aeruginosa*, nutritional parameters, medications, exacerbations in last year, CF complications, and genotype, was obtained at each centre.Infection with *P. aeruginosa* was defined using Leeds criteria (Lee, Brownlee et al. 2003), classifying patients as 1) never, 2) free of infection (none

positive in the last year), 3) intermittent (≤ 50% of cultures in last year), 4) chronic (>50% of cultures are positive or presence of mucoid *P. aeruginosa*).

Some paediatric patients had been taking chronic antibiotics prior to the sample being collected. Five children were on long term azithromycin therapy and only one child was on long term flucloxacillin treatment.

The table below highlights the data from the Dublin group (Table 3.1) and it shows the baseline characteristics of the paediatric patients in the study group. 74.5% of the patients studied, had the common DF 508 homozygous mutation, while 96.5% of patients studied had a least one DF 508 allele present. Genotype testing was confirmed by both sweat testing and CF mutation analysis.

Characteristic	Male	Female
Gender	34	24
Mean age -yr	7.35	6.62
Control mean -yr	3	1
Ethnicity- %	Caucasian- 100	Caucasian-100
Weight < 6 yrs-Kg Mean 3.21 years	19.2	15.03
Weight > 6 yrs-Kg Mean 14.5 years	43.79	40.73
Growth Chart< 6yrs Mean 3.21 years	Height- 25th centile-100.96 cm Weight- 50th centile- 16.34Kg	Height-25th centile-97.35cm Weight-50th centile-15.9Kg
BMI> 6yrs Mean 14.5 years	17.98	17.3

FEV_1 L/ FEV_1 %	1.99 L +- 0.91 / 74.9% +- 24.1	1.5L/ 56.5% +- 7.78
Bronchoalveolar lavage sample number (extra sample obtained at subsequent visit)	22 (4)	15(2)
Sputum samples (extra sample obtained at subsequent visit)	11(2)	5
Exacerbation sputum samples(extra sample obtained at subsequent visit)	6(2)	3

Table 3.1: Baseline characteristics of paediatric patients enrolled in the study from 2010-2013.

BAL samples taken from children under 6 years of age were sent to the clinical laboratory for culture. The majority of samples in routine hospital culture grew *S. aureus, P.aeruginosa* or had no growth (Table 3.2). All samples were sent for anaerobic culture, however few samples had growth and the exact genus and subtype was not always easy to identify.

The DNA of each isolate was also sent for further second generation sequencing. This was extracted by the FAST prep technique. Samples were stored in the -80°C freezer and these were then shipped on ice for further sequencing using 16S ribosomal RNA sequencing. More species have been isolated from the analysis then by simple culturing the plates. rRNA sequences and especially the 16S rRNA sequencing represent the most important current targets of study in bacterial evolution and ecology, including the determination of

phylogenetic relationships among taxa, the exploration of bacterial diversity in the environment and the quantification of the relative abundance of taxa of various ranks.

Both culture and 16S ribosomal techniques together are both useful in identifying both the bacterial taxonomy and phylogeny. One of the most attractive potential uses of 16S rRNA gene sequence informatics is to provide genus and species identification for isolates that do not fit any recognised profiles. It is useful for strains generating only a "low likelihood" or "acceptable" identification according to commercial systems, or for taxa that are rarely associated with human infectious diseases (Janda and Abbott 2007). From the following tables (Tables 3.2-3.4, 3.7-3.10), there is a detailed result of both aerobic and anaerobic bacteria and examples of the more comprehensive report from 16 S rRNA sequencing.

Few of the samples from BAL were colonised with SMG group or other commensals on the 16S r RNA sequencing. There was a further problem with the misreading of *P.aeruginosa* as *P.otitidis* on all samples and an underrepresentation of the amount of *S.aureus* present. This probably reflects the fact that both these bacteria have closely related 16S r RNA clones and this introduced microvariation arifacts on reading the samples, giving a false negative on 16S. This error is currently being investigated by biotechnicians at UNC (Table 3.2).

Study ID	Hospital culture	Anaerobic culture	16S rRNA assessment						
D016	none	none	*N. subflava*	*H. aegyptius*	*N. subflava*	*N. subfava*			
D017	none	none	*N. subfava*	*N. subflava*	*N. subflava*	*S. epidermidis*	*Abiotropha defectiva*		
D021	*P.aeruginosa, S.aureus*	none	*P. otitidis*	*S. aureus*					
D034	*P.aeruginosa, S.aureus*	yes	*P. otitidis*	*H. parainfluenzae*	*S. aureus*	*P. otitidis*	*S. anginosus*		
D035	*S.aureus*	none	*S. mitis*	*S. flexneri*	*S. aureus*	*S. aureus*			
D046	none	none	*H. huttiense*	*H. huttiense*	*S. maltophilia*	*H. huttiense*			
D047	none	none	*H. huttiense*	*H. huttiense*					
D048	*P.aeruginosa*	yes	*P. otitidis*	*P. otitidis*	*S. mitis*	*N. subflava*	*S. salivarius*	*S. sanguinis*	*S. sanguinis*
D049	*P.aeruginosa*	yes	*P. otitidis*	*S. maltophilia*	*S. pseudopneumoniae*	*C. diphtheriae*	*G. sanguinis*	*S. pseudopneumoniae*	
D059	*P.aeruginosa*	none	*P. otitidis*	*H. huttiense*	*S. salivarius*				
D060	none	none	*S. pseudopneumoniae*	*S. vestibularis*	*C. pseudodiphtheriticum*	*N. polysaccharea*	*S. salivarius*	*V. parvula*	
D070	none	none	*S. mitis*	*H. influenzae*	*A. massiliensis*				
D071	none	none							
D072	none	none	*N. subflava*	*S. oralis*	*H. influenzae*	*H. influenzae*			
D076	none	none	*N. subflava*	*R. mucilaginosa*	*N. polysaccharea*	*S.. flexneri*	*H. parainfluenzae*	*S. salivarius*	*P. nanceiensis*
D077	none	none	*S.. flexneri*	*Micrococcus luteus*	*F. periodonticum*				
D 078	none	none	*R. dentocariosa*	*S. mitis*					
D083	*none*	*none*	*R. dentocariosa*	*S. pseudopneumoniae*	*N. lactamia*				
D084	*none*	yes	*N. polysaccharea*	*N. subflava*	*S. anginosus*				
D095	*S.aureus*	none	*S. aureus*	*S. aureus*	*H. influenzae*				
D104	none	none	*Rothia mucilaginosa*	*G. haemolysans*	*S. mitis*	*S. salivarius*			
D116	none	yes	*N. subflava*	*S. pneumoniae*	*N. polysaccharea*	*S. maltophilia*	*S. salivarius*	*Prev nigrescens*	
D117	*Rothia*	yes	*S. sanguinis*	*S. salivarius*	*Rothia mucilaginosa*	*N. subflava*	*Rothia mucilaginosa*	*S. mitis*	*H. parainfluenzae*
D118	*P.aeruginosa*	P.melaninogenica	*P. otitidis*	*P. otitidis*	*S. salivarius*	*P melaninogenica*			
D123	none	none	*H. parainfluenzae*						
D124	none	none							
D125	*S.aureus*	none	*S. aureus*	*N. subflava*	*N. subflava*	*H. influenzae*	*Prev nanceiensis*	*Porphyromonas catoniae*	
D126	none	none	*N. subflava*	*S. sanguinis*	*Rothia mucilaginosa*	*Parvimonas micra*			

Table 3.2: BAL samples from CF stable children < 6 years in Crumlin 2010-2013. A few of the anaerobic culture plates grew bacteria; however, it was not possible to identify them completely. 16S r RNA sequencing misread *P. aeruginosa* as *Pseudomonas otitidis* (*P. otitidis*) in all samples.

Study ID	Routine hospital culture	Anaerobic culture	16S rRNA assessment
D062	none	none	Results awaited
D063	none	none	Results awaited
D132	none	none	Results awaited
D124	none	none	Results awaited
D142	none	none	Results awaited

Table 3.3: Culture and sequencing data from control patients (children < 6years of age) and these BAL samples were collected at Crumlin 2010-2013. All control patients underwent a bronchoscopy for suspected tracheobronchomalacia or because of haemoptysis. No growth was seen on routine hospital culture or anaerobic culture. Results are awaited on 16S r RNA assessment.

Control samples were taken from children, < 6 years of age and who underwent bronchoscopy for non infectious cause (Table 3.3). No bacteria grew from hospital or anaerobic culture on these patients. 16S r RNA assessment is still outstanding as priority was initially given to samples from CF patients.

Study ID	Hospital culture	Anaerobic culture	16S r RNA assessment					
D086	P.aeruginosa	None	*R. dentocariosa*	*S. aureus*	*S. mitis*	*P. otitidis*	*P. otitidis*	*L. rhamnos*
D087	P.aeruginosa	None	*S. aureus*	*P. otitidis*	*S. aureus*			
D088	P.aeruginosa	None	*P. otitidis*	*S. anginosus*				
D089	M.absesssus,MRSA	None	*S. aureus*	*S. salivarius*	*S. salivarius*	*Fusobacterium periodonticum*		
D090	M.abscessus, MRSA	None	*S. aureus*	*N. subflava*	*N. subflava*	*S. aureus*	*S. mitis*	
D091	MRSA	None	*S. aureus*	*A. naeslundii*	*P. otitidis*	*S. aureus*	*Gemella sanguinis*	*P.melaninogenica*
D092	none	None	*S. aureus*	*S. salivarius*	*S. anginosus*	*P.melaninogenica*		
D108	S.aureus, Achromobacter	None	*Rothia dentocariosa*	*Achromobacter xylosoxidans*	*S. mitis*	*Achromobacter xylosoxidans*	*S. salivarius*	*S. mitis*
D109	none	None	*A. naeslundii*	*S. mitis*	*Pseudomonas beteli/hibiscola*	*N. subflava*	*A. naeslundii*	*S. salivarius*
D112	A.fumigatus	None	*Rothia dentocariosa*	*S. aureus*	*Rothia dentocariosa*	*Gemella sanguinis*	*P. nigrescens*	*P. histicola*
D121	none	None	*S. aureus*	*S. aureus*	*S. aureus*	*S. epidermidis*	*Rothia*	

							mucilaginosa	
D122	none	None	*S. aureus*	*S. aureus*	*S. aureus*	*S. aureus*		
D128	none	None	*Rothia dentocariosa*	*S. anginosus*	*Enterococcus faecium*	*Enterococcus faecium*	*Enterococcus faecium*	*Enterococcus faecium*
D133	P.aeruginosa	None						
D134	none	None						
D141	none	None						

Table 3.4: Culture and 16S ribosomal RNA sequencing data from stable children 12-18 years. Sputum samples were collected at Temple Street and Crumlin hospitals 2010-2013. Few anaerobes were detected on culture and sequencing. The ribosomal sequencing was able to differentiate MRSA from *S.aureus* and it failed to identify the samples with *M.abscessus*.

Sputum from children was only taken from those that were over 12 years of age, as this group were able to produce sputum in sufficient quantities for further analysis (Table 3.4). However, this group were also likely to be sicker and have lower FEV_1 values. From the analysis, in this group, n=17, one child grew Streptococcus mitis, a facultative anaerobe from the sequencing and this may be as a result of oral contamination. 16S ribosomal RNA sequencing failed to differentiate between methicillin resistant *S. aureus* (MRSA) and *S.aureus*. It also failed to recognise *Mycobacterium abscessus.* This illustrates certain drawbacks with using 16S r RNA sequencing. It is useful in regards to bacterial classification; it has a low phylogenetic power and species level and is unable to detect certain genra (Janda and Abbott 2007).

We also analysed the BAL cell differential from the stable CF children < 6years, by cytopsin.Distinct patterns of inflammation were seen in the sample cohort analysed (n=40), a high neutrophil burden was seen in this group

suggesting that even in this young population infection and inflammation is taking place (Table 3.3).

Differential Cell Count (%)

Patient ID	Macrophage	Neutrophil	Lymphocytes	Eosinophils	Bronchial epithelial cells	Squamous cells	Oral squamous cells	Red cells
D 16	80	16	3	Rare				
D 17	76	33		Rare		Rare		
D 21	55	44		Rare				
D 35	42	54		2		2		
D 46	88	11	Rare					
D 47	56	43						
D 48	93	6			Rare		Rare	
D 49	95	5					Rare	
D 59	80	19					Rare	
D060	84	12			3		1	
D 62	95		1		2	2		
D 63	45	55	2					
D 70	87	12	1					
D71	37	62	1					
D72	32	66	1				1	
D76	90	3			3			
D 77	73	26	Rare					
D 78	86	14						
D 83	64	36						
D 84	83	14			Rare			
D95	50	40			10			
D 104	90	3	6		Rare			
D 117	78	20	1	Rare	Small No.			
D116	56	40	3					
D 118	8	90%					Rare	
D 123	42	57			1			
D 124	62	36			Rare			
P68	78	20	1	Rare	Small No.			
P78	10	90						
P86	30	66	1		3			

P88	55	44	1				
P98	55	44	1		3	2	
P99	90	10					
P101	90	8	1		1		
P102	83	14	1	1	1		
P105	95		3		2		
P108	80	18	1		1		

Table 3.5: Cytospin results from CF BALF stable samples show that there is a high neutrophil and macrophage burden in patients with the young patients with CF.

3.1.2 Adult recruitment

Adult patients were asked to participate in the study from 2010 until the end of recruitment in 2013. Adult patients over 18 years old attending the OPD were initially considered however there were certain exclusion criteria which prevented patients from being recruited. Prior to commencement of the study, ethics approval had been given to Beaumont hospital for this study. The exclusion criteria were that; patients were only allowed give an initial stable sample of sputum, if they had been off antibiotics for the preceding 1 month. Patients currently on oral or IV antibiotics were excluded at that time from the study; however they could be included 4 weeks following the stop date of the antibiotics. Other patients excluded were lung transplant recipients, pregnant females and patients already on other antibiotic trials.

Control subjects were also recruited; these were healthy adult volunteers and were age matched controls. They were asked to give sputum if possible or were asked to give an induced sputum sample. The induced sample was given following pre-treating the subject with salbutamol inhaler and then giving the subject while supervised by physiotherapist and doctor, 3ml dose of 3% hyper

tonic saline given with an ultrasonic nebuliser for fifteen minutes. The sample was then collected and placed in an anaerobic bag. The exclusion criteria for the control patients included all patients with asthma, or those who had bronchiectasis or frequent infections. Patients with immunodeficiencies, those currently on antibiotic therapy, pregnant females or those that have any other chronic respiratory disorder were all excluded from the study.

When patients were recruited they were enrolled on either the cross sectional or longitudinal arm of the study. If patients enrolled on the cross sectional arm, they were asked to read the information leaflet, sign a consent form and give a once off sputum, blood and urine sample. Data was recorded after the visit and entered onto the database. Biological samples were collected and processed immediately in the laboratory.

Patients were also asked to consent to being enrolled on the longitudinal arm of the study. Patients were asked to give sputum, blood and urine samples both at stable (S1), pre (E1) and post exacerbation (E2) and then at another stable interval at least 30 days apart (S2). Data again was recorded after each visit and entered into the database. Biological samples were collected and processed in the laboratory. The exacerbation criteria were similar to the paediatric cohort.

A total of 83 adults' disease subjects and 4 control patients were recruited to the study. 61% of the patients recruited are male. Both male and female patients are relatively similarly age matched, 25.78 years and 27.65 years respectively (Table 3.6). The control subjects were patients recruited from

outpatients with a no known respiratory history. The samples given were induced samples as described previously.

Characteristic	Male	Female
Gender	51	32
Controls	0	4
Age-mean yrs +/- SD	25.78 +/- 7.13	27.65 +/- 8.52
FEV_1 stable	2.32L +/- 1.35 L, 56%	2.4L +/- 0.69, 56.9%
FEV_1 exacerbation	1.63L +/-0.61 L, 38.5%	1.53 L +/- 0.21 L, 49%
Longitudinal follow up	14	12

Table 3.6: Baseline characteristics of stable adult patients recruited from 2010-2013

In our control patient group, all samples given were from induced sputum. Controls were well and were age matched controls (Table 3.7). There are anaerobic pathogens detected in the samples, *S. mitis, S. sanguinis and V.paruvula* occurring in 10% of the sample group. Total average viability counts are 1.1 $x10^7$, 2.9 $x10^4$, and 2.3 $x10^7$ respectively.

Study ID	Routine Hospital Culture	Anaerobic Culture	16S rRNA assessment							
D110	none	none	*R. mucilaginosa*	*S. cristatus*	*A. naeslundii*	*H. parainfluenzae*	*S. mitis*	*R. dentocariosa*	*V. parvula*	
D111	none	none	*R. dentocariosa*	*S. sanguinis*	*S. salivarius*	*R. dentocariosa*	*R. dentocariosa*	*S. salivarius*	*A. odontolyticus*	*Catonella morbi*
D113	none	none	*S. mitis*	*Neisseria subflava*	*A. naeslundii*	*R. dentocariosa*	*H. parainfluenzae*	*S. salivarius*	*P. oris*	*P. denticola*
D115	none	none	*Neisseria subflava*	*S. parasanguinis*	*S. parasanguinis*	*H. parainfluenzae*	*A. segnis*	*Parvimonas micra*	*A. naeslundii*	*V. parvula*

Table 3.7: Culture and 16 S ribosomal sequencing data on the control adults. The majority of bacteria detected in adult control samples remain oral commensals. No growth was seen on routine hospital or anaerobic culture.

In the stable adult group (S1), 40% of growth was due to *P.aeruginosa.* 24 patients grew both mucoid and non-mucoid *Pseudomonas.* 21% grew methicillin sensitive *S. aureus* (MSSA), 1% growth of MRSA, 3% growth of *Stenotrophomonas maltophilia (S. maltophilia),* 7% growth of *Aspergillus fumigatus (A. fumigatus) and 23%* growth of *Candida albicans.* The other bacteria are made up of *Ralstonia, Burkholderia* species, *Achromobacter*, *Haemophilus influenza* and *E-coli* (Table 3.8).

Study ID	hospital culture		16S RNA assessment								
D001	*B.gladioli*										
D002*	*P.aeruginosa, S.aureus,S.maltophilia*	yes	*S. salivarius*	*R. mucilaginose*	*S. parasanguinis*	*S. parasanguinis*	*M. caterrhalis*	*S. parasanguinis*	*M. catarrhalis*	*S. infantis*	*P. salivae*
D003	*P.aeruginosa,C.albicans*										
D004*	*P.aeruginosa*	none	*P. otitidis*	*S. salivarius*	*P. otitidis*	*P. otitidis*	*P. otitidis*	*S. infantis*	*P. otitidis*	*S. salivarius*	*S. salivarius*
D005*	*S.maltophilia,A.fumigatus*										
D006	*P.aeruginosa*	none	*P. otitidis*	*P.otidis*	*P. otitidis*	*P. otitidis*	*P.otidis*	*P.otidis*	*A. naeslundii*	*S. epidermidis*	*P. otitidis*
D007*	*none*										
D008	*P.aeruginosa, C.albicans*	none	*results awaited*								
	P.aeruginosa, C.albicans										
D010*	*P.aeruginosa, C.albicans*	yes	*S. parasanguinis*	*S. salivarius*	*S. parasanguinis*	*S. sanguinis*	*P. otitidis*	*L. gastricus*	*G. adiacens*	*S. anginosus*	*E. faecalis*
D011	*S.aureus, A.fumigatus*										
D012	*P.aeruginosa, C.albicans*	none	*R. mucilaginosa*	*R. dentocarlosa*	*S. mitis*	*S. mitis*	*P. otitidis*	*L. rhamnos*	*S. mitis*	*S. mitis*	*L. rhamnos*
	S.aureus,C.albicans										
D014	none	yes	*S. salivarius*	*H. huttiense*	*S. cristatus*	*S. maltophilia*	*S. parasanguinis*	*S. salivarius*	*R. mucilaginosa*	*S. cristatus*	*P. melaninogenica*

D015	P.aeruginosa, C.albicans										
D018	P.aeruginosa,A.fumigatus	yes	R. dentocarlosa	P. otitidis	S. vestibularis	Actinomyces neaslundii	P. otitidis	S. salivarius	S. salivarius	G. adiacens	S. parasanguinis
D019*	P.aeruginosa, A.fumigatus										
D020*	P.aeruginosa,S.aureus	none	R. mucilaginosa	P. otitidis	P. otitidis	R. dentocariosa	H. huttiense	S. intermedius	S. intermedius	P. otitidis	P. otitidis
D022	P.aeruginosa, C.albicans										
D023*	P.aeruginosa, A.fumigatus,C.albicans	yes	S. anginosus	S. salivarius	P.otidis	P. otitidis	P. otitidis	P. otitidis	P. otitidis		
D024	P.aeruginosa, A.fumigatus,C.albicans										
D025	P.aeruginosa, C.albicans, S.aureus	none	S. salivarius	P. otitidis	S. mitis	P. otitidis	P. aeruginosa	P. otitidis			
D026	P.aeruginosa,S.aureus, C.albicans										
D027	P.aeruginosa, C.albicans	none	P. otitidis	S. salivarius	P. otitidis	G. adiacens	P. otitidis	P.otidis	P. otitidis	P. otitidis	S. pyogenes
D028	P.aeruginosa, C.albicans										
D029	P.aeruginosa, C.albicans	yes	P. otitidis	S. anginosus	S. maltophilia	B. subtilis	S. pseudopneumoniae	P.otidis	S. salivarius		
D030	P.aeruginosa, C.albicans										
D031	P.aeruginosa,A.fumigatus	none	S. lugdunensis	P. otitidis	Yeast	E. faecalis	Yeast	P. otitidis	S. lugdunensis	E. faecalis	P. otitidis
D032*	S.aureus,C.albicans										
D033	P.aeruginosa	yes	S. maltophilia	P. otitidis	P. otitidis	P. otitidis	R. mucilaginosa	S. infantis	P. otitidis	S. parasanguinis	S. aureus
D036*	P.aeruginosa, C.albicans										
D037	P.aeruginosa	none	no results								
D038	C.albicans										
D039	S.aureus, Ralstonia	none	P. otitidis	S. aureus	S. maltophilia	S. salivarius	E. faecalis	S. maltophilia	H. huttiense	H. huttiense	P. otitidis
D040	A.fumigatus, S.aureus,C.albicans										
D041	S.aureus,S.maltophilia, Ecoli	P.melaninogenica	S. mitis	S. anginosus	Shig. flexneri	Shig. flexneri	Shig. flexneri	P. nigrescens			
D042	none										
D043	P.aeruginosa, S.aureus,A.fumigatus, H.influenzae	yes	Shig. flexneri	S. australis	S. anginosus	S. australis	S. sanguinis				
Do44	P.aeruginosa, S.aureus, C.andida										
D045	S.aureus,C.albicans,B.cepaciae	yes	S. mitis	S. angin	P. salivae						

				osus							
D050	P.aeruginosa,S.maltophilia, A.fumigatus, C.albicans	yes									
D051*	S.aureus, A.fumigatus, C.albicans	none	S. aureus	H. huttiense	H. huttiense	H. huttiense	S. anginosus				
D052	P.aeruginosa, S.aureus	yes									
D053	P.aeruginosa	none	P. otitidis	P. otitidis	S. maltophilia	S. salivarius	A. odontolyticus	P. otitidis	L. rhamnos	S. mitis	P. otitidis
D054	S.aureus, C.albicans	yes	S. aureus	S. aureus	S. salivarius	P. otitidis	S. aureus	A. odontolyticus	S. aureus	S. australis	S. salivarius

D055	P.aeruginosa, S.aureus										
D056*	P.aeruginosa, A.fumigatus, C.albicans	none	P.otidis	S. salivarius	P. otitidis	P. otitidis	P. otitidis	S. salivarius	A. naeslundii	P. otitidis	P. otitidis
D057	S.aureus										
D058	S.aureus, C.albicans	none	N. subflava	P. otitidis	P. otitidis	H. huttiense	S. salivarius				
D061	P.aeruginosa										
D064	P.aeruginosa, S.aureus	none	no results								
D065	none										
D066*	P.aeruginosa, C.albicans, Achromobacter	none	no results								
D067	P.aeruginosa, S.aureus										
D068	P.aeruginosa, S.aureus	P.nigrescens	P. nigrescens								
D069	P.aeruginosa, C.albicans										
D073	P.aeruginosa, S. aureus, C.albicans	none	S. aureus	P. otitidis	A. naeslundii	P. otitidis	P. otitidis	S. salivarius	V. parvula		
	P.aeruginosa, S. aureus, C.albicans										
D075	P.aeruginosa, C.albicans	none	S. aureus	S. salivarius	S. aureus	S. salivarius	V. dispar	S. aureus			
D079	P.aeruginosa, C.albicans										
D080	P.aeruginosa, C.albicans	yes	S. agalactiae	S. mitis	S. agalactiae	L. pentosus					
D081	P.aeruginosa, S.aureus, C. albicans, S.maltophilia										
D082	P.aeruginosa, C.albicans	none	P. otitidis	P. otitidis	P. otitidis	P. otitidis	P. otitidis				
D085	S.aureus, C.albicans										
D093*	P.aeruginosa,A. fumigatus, C.albicans	yes	S. aureus	S. aureus	S. pseudopneumoniae	P. otitidis	S. mitis				
D094	P.aeruginosa, S.aureus, A.fumigatus										
D096	S.aureus, C.albicans	none	R. dentocariosa	S. vestibularis	A. naeslundii	S. anginosus	L. crispatus				
D097*	S.maltophilia, C.albicans										
D098	Ralstonia	none	S. anginosus	S. salivarius	R. mannitolilytica	P. salivae					
	S.aureus										
D100	S.aureus	yes	S. aureus	S. aureus	S. mitis	L. wadei					

D101	*S.aureus, C.albicans, B. Cepacine*										
D102	*P.aeruginosa, C.albicans*	yes	*S. salivariu s*	*R. dentocar losa*	*S. paras anguin is*	*S. salivariu s*	*S. paras angui nis*	*R. dentocari osa*	*R. mucilagi nosa*	*S. salivari us*	*E. saburreu m*
D103	*S.aureus, C.albicans*										
D105	*P.aeruginosa, C.albicans*	yes	*P. otitidis*	*P. otitidis*	*P. otitidis*	*A. naeslun dii*	*S. saliva rius*	*S. constella tus*	*P. oris*	*P. nigresc ens*	*P. nigresce ns*
D106	*P.aeruginosa, Achromobacter*										
D107	*S.aureus*	none	*S. aureus*	*S. infantis*	*A. naeslu ndii*	*S. aureus*	*S. aureu s*				
D114	*S.aureus, C.albicans*										
D115	none	yes	*N. subflava*	*S. parasan guinis*	*S. paras anguin is*	*H. parainflu enzae*	*A. segni s*	*P. micra*	*A. naeslund ii*	*V. parvula*	
D119	*P.aeruginosa, C.albicans*										
D120	*S.aureus, MRSA, C.albicans*	yes	*R. dentoca riosa*	*R. dentocar losa*	*H. influen zae*	*S. sanguini s*	*P melan inoge nica*				
	S.maltophilia, C.albicans										
D129	*S.aureus, C.albicans*	none	*S. aureus*	*S. aureus*	*R. dentoc ariosa*	*N. subflava*	*R. dento carlos a*	*V. atypica*			
D130	*P.aeruginosa, C.albicans*										
D131	*S.maltophilia, A.fumigatus*	none	results awaited								

Table 3.8: Stable CF patients' 16S RNA sequencing data from Beaumont patients 2010-2013.

Study ID	hospital culture	anaerobic culture	16S RNA assessment				
D001	*S. aureus*	*P.melaninogenica*	P.melaninogenica	S. mitis	N. bacilliformis	S. anginosus	R. dentocariosa
D002*	*P.aeruginosa*	*P.melaninogenica*	P.melaninogenica	S. vestibularis	S. pseudopneumoniae	P. otitidis	Shig. flexneri
D003*	*H.influenzae*	SMG	*H. influenzae*	*S. oralis*	*S. parasanguinis*	*S. sanguinis*	*S. pseudopneumoniae*
D004*							
D005*							
D006*	*S.maltophilia*	P.melaninogenica	S. maltophilia	P. denticola	P.melaninogenica	S.mitis	
D007*							
D008*	*P.aeruginosa*		P. histicola	P.otitidis			
D009*	*P. aeruginosa*	none	R. mucilaginosa	R. mucilaginosa	P. otitidis	Granulicatella adiacens	
D010*							
D011*	*S. aureus*	P.nigrescans	S. anginosus	S. salivarius	P. nigrescens	Fusobacterium nucleatum	
D012*	*P.aeruginosa*	SMG	P. otitidis	H. huttlense	S.mitis	P. otitidis	Brevibacteria frigoritolerans
D013							
D014							
D015							
D018	*P.aeruginosa*	SMG	P. otitidis	A. naeslundii	P. otitidis	S. anginosus	S. salivarius
D019*	*P. otitidis*	none	S. salivarius	S. salivarius	S. salivarius	P. otitidis	P. otitidis
D020*	*P. aeruginosa*	none	P. otitidis	S. salivarius			
D022	*P.aeruginosa*	none	R. mucilaginosa	L. gastricus	P. otitidis	S. mitis	Peptostreptococcus anaerobius
D023*	*P. aeruginosa*	none	P. otitidis	P. otitidis	P.melaninogenica		
D024							
D025	*P. aeruginosa*	yes	S. anginosus	S. anginosus	P. otitidis	P. otitidis	
D026	*S. aureus*	none	S. mitis	N. subflava	P. otitidis	S. aureus	V. atypica
D027							
D028	*P. aeruginosa*	none	R. dentocariosa	R. dentocariosa	P. otitidis	Gemella haemolysans	Atopobium rimae

D029	*P. aeruginosa*	none	P. otitidis	P. otitidis	Prev. oralis	
D030	*P.aeruginosa*	none	N. subflava	Strep. gordonii	P. otitidis	A. odontolyticus
D031						
D032*						
D033						
D036*						
D037						
D038						
D039	*S. aureus*	P.nigrescens	S. salivarius	P. nigrescens	P. nigrescens	A. odontolyticus
D040						
D041	*none*	SMG		S. anginosus		

Table 3.9:Culture an16S ribosomal sequencing data for exacerbation group, pre-antibiotics (E1) of adult CF patients recruited at Beaumont hospital 2010-2013.

In the E1 patient group, routine culture and anaerobic culture data detected anaerobes in 2 of the patient group. The full data set from the 16S r RNA sequencing is still pending, however the majority of anaerobic isolates were from 10 patients and these included, *P. nigrescens*, *P. melaninogenica and F. nucleatum* as outlined in Table 3.9. In the E2 patient group,less numbers of anaerobes are seen in both the culture data and 16S r RNA sequencing and these are mainly from SMG group and *P. melaninogenica* (Table 3.10). 55% growth of oral commensals .23% *Pseudomonas aeruginosa,* and remaining are anaerobes. The sequencing used did not detect Staph Aureus in the sample group. From both culture and sequencing data from the exacerbation group, a more complicated number of microbes are discovered with the aid of 16 S ribosomal sequencing. Many of the anaerobes were still present after routine treatment of the predominant bacteria that grew on the aerobic plate (Table 3.10). Patients in the longitudinal cohort were all non-smokers. If they were

colonised by routine hospital culture with *P.aeruginosa*, they were on oral azithromycin and alternating months of nebulised colomycin or tobramycin. Antibiotics used were dependant on the resistant patterns of the patient's hospital culture. If patients were colonised with *P.aeruginosa*, they were likely to be on two antipseudomonal cover, one of which included either ceftazidime, piperacillin/tazobactam or meropenem for 14 days. These antibiotics have good cover against anaerobes.

Study ID	hospital culture	Anaerobic Culture	16S rRNA sequence								
D001		none detected									
D002		none detected									
D003		none detected									
D004		none detected									
D005		none detected									
D006	P.aerugi nosa	none detected	*P. otitidis*	*P. otitidis*	*P. otitidis*	*P. otitidis*					
D007		none detected									
D008		none detected									
D009	*P. aerugino sa*	none detected	*P. otitidis*	*Micrococcus luteus*	*P. otitidis*	*Granulic atella adiacens*					
D010		none detected									
D011	*S. aureus*	none detected	*S. aureus*	*S. aureus*	*S. anginosus*	*P. salivae*	*Fusobact erium nucleatu m*				
D012	none	S.mitis									
D013		none detected									
D014		none detected									
D015		none detected									
D018	none	none detected	*Enteroco ccus faecalis*	*S. salivarius*	*Enterococcu s faecalis*	*Enteroco ccus faecalis*					
D019	none	none detected									
D020	none	none detected									
D022	*P.aerugi nosa*	none detected	*Enteroco ccus faecalis*	*P. otitidis*	*Enterococcu s faecalis*						
D023	*P. aerugino sa*	none detected									
D024		none detected									
D025	*P. aerugino sa*	none detected	*P. otitidis*	*S. salivarius*	*S. pseudopneu moniae*						
D026	*S. aureus*	none detected	*P. otitidis*	*H. huttiense*	*H. huttiense*						
D027		none detected									
D028	*P. aerugino sa*	none detected	*P. otitidis*	*S. pseudopneu moniae*	*P. otitidis*	*P. otitidis*	*P. otitidis*	*P. otitidis*	*L. parac asei*	*Pre v. ons*	*S. constell atus*
D029	*P. aerugino sa*	S.mitis	*P. otitidis*	*P. otitidis*	*P. otitidis*	*P. otitidis*	*P. otitidis*	*S. mitis*			
D030		S.mitis	*N. subflava*	*S. mitis*	*S. salivarius*	*N. subflava*	*S. salivarius*	*P. nigresc ens*	*S. mitis*	*P. otiti dis*	*P. nigresc ens*
D031		none detected									
D032		none detected									
D033		none detected									
D036		none detected									
D037		none detected									
D038		none detected									
D039	*S. aureus*	none detected									

D040		none detected				
D041	*Shig. flexneri*	none detected	*Shig. flexneri*	*S. mitis*	*Shig. flexneri*	*S. infantis*

Table 3.10: Culture and 16S ribosomal sequencing data for post exacerbation group (E2) adult CF patients recruited at Beaumont hospital 2010-2013.

Study ID	hospital culture	anaerobic culture	16S rRna assessment						
D001	*S. aureus*	none detected							
D002*	*S.aureus*	P.melaninogenica	*R. mucilaginosa*	*S. aureus*	*S. oralis*				
D003	*P.aeruginosa*	none detected	*P. otitidis*	*R. dentocariosa*	*P. otitidis*	*S. salivarius*			
D004*		none detected							
D005*		none detected							
D006		none detected							
D007*		none detected							
D008		none detected							
D009	*P. aeruginosa*	none detected	*P.otitidis*						
D010*		none detected							
D011	*S. aureus*	none detected	*N. subflava*	*S. anginosus*	*S. sanguinis*	*S. aureus*	*V. atypica*		
D012	*none*	none detected	*S.mitis*						
D013		none detected							
D014		none detected							
D015		none detected							
D018		none detected							
D019*	*P. otitidis*	*P.melaninogenica*	*R. mucilaginosa*	*A. odontolyticus*	*R. mucilaginosa*	*P. otitidis*	*P.melaninogenica*	*S. parasanguinis*	
D020*	*P. aeruginosa*	none detected	*P. otitidis*	*Enterococcus faecalis*	*Enterococcus faecalis*	*P. otitidis*	*P. otitidis*		
D022		none detected							
D023*	*P.aeruginosa*	none detected	P. otitidis						
D024		none detected							
D025	*P. aeruginosa*	none detected	*P. otitidis*	*P. otitidis*	*R. dentocariosa*	*P. otitidis*	*P. otitidis*	*S. anginosus*	*P. saliva e*

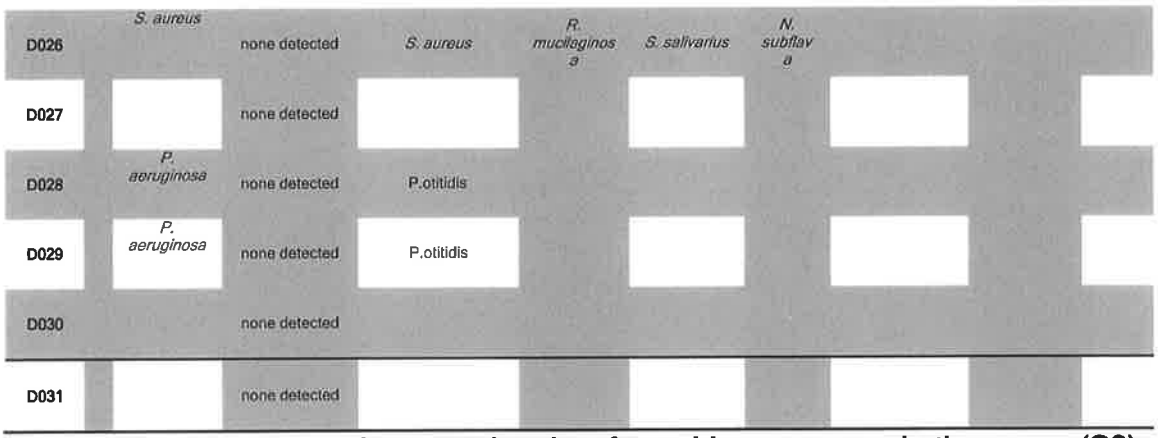


Table 3.11:16S ribosomal sequencing data for stable post exacerbation group (S2) adult CF patients recruited at Beaumont hospital between 2010-2013

In many of the patients, 19/27, the same anaerobe persisted or a different anaerobe was present post treatment. Similar studies from our collaborators have shown(Fodor, Klem et al. 2012), a stable overall structure in airway, despite antibiotic treatment, however, long term changes in microbial community richness are likely to be associated with a slow decline in CF function. 62% (28/45) of PA positive adult patients at both stable and exacerbation points grew anaerobes and the most common grown *P.melaninogenica, P.nigrescens, F. nucleatum and S. mitis.* 34 % (20/58) of PA negative stable adult patients had anaerobes but they grew predominantly *S. anginosus, P. melaninogenica* and *F.nucleatum*.

3.1.3 Discussion

This study has highlighted some of the current limitations associated with standard diagnostic microbiology and how there can be shortcomings in the current techniques used in laboratories (Kerem, Conway et al. 2005).

Conventional culture methods are complicated by the problem of slow growing microbes, being overgrown by abundant, faster growing organisms. There have been studies highlighting this problem and the fact that many new and potentially harmful pathogens are currently being overlooked by current practice (Tunney, Field et al. 2008, Ghegan, Wise et al. 2009). There has been a considerable advance in next-generation sequencing over the last few years. Anaerobes have only come to light now that there is a greater interest in the CF microbiome. Many strict anaerobes are antibiotic resistant (Tunney, Field et al. 2008, Worlitzsch, Rintelen et al. 2009, Pattison, Rogers et al. 2013).

In paediatric samples, molecular detection was responsible in the stable population of increasing the diagnostic yield, 2 patients had *Pseudomonas aeruginosa* and 1 patient had *Stenotrophomonas maltophilia* that were not detected by routine culture. 14% of CF stable patients in this population had anaerobes and these were predominantly *Streptococcus mitis* which is regarded as a facultative anaerobe, not known to be responsible for pathogenicity. Harris and colleagues, found anaerobes which were mostly oral commensals in their group and suggested that anaerobes could be the cause of inflammation and clinical deterioration without any new pathogen found (Harris, De Groote et al. 2007).Similarly with the older children, sputum at stable and exacerbation points produced very little anaerobes. Adult stable CF patients grew few anaerobes by routine culture but by 16S r RNA sequencing, the majority found were *P. melaninogenica* and *S. sanguinis*. However from the age matched control group, *S. mitis* and *S. sanguinis*, two potentially less virulent strains were isolated. Adult CF patients during exacerbations, produced a lot more diverse bacteria, *P.*

melaninogenica, V. parvula, F. nucleatum and this was more likely if they were initially *P. aeruginosa* positive. This potentially could be due to the fact that *P. aeruginosa* can influence virulence factor genes of pathogens potentially present in the oropharyngeal flora strains and make them more virulent (Duan K 2003).

Therefore oropharyngeal bacteria likely participate in disease progression and are probably an underestimated emerging cause of CF lung pathology that should be considered in the antibiotic strategy(Bittar, Richet et al. 2008). This phenomenon of inter-cellular communications is also important in the constitution of the biofilms (Singh, Schaefer et al. 2000).

None of the control or disease patients recruited were smokers. 65 patients from the adult group and 6 from the paediatric group were on either nebulised antibiotics or azithromycin. These patients were also likely to be colonised with *P.aeruginosa* as these antibiotics are used for treatment. There were higher numbers of anaerobes associated with these patients, which may reflect that these patients are sicker and so the anaerobes may have a role in creating a more inflamed environment. However, this relationship is still being analysed by our collaborators.

In conclusion, the amplification of 16s DNA presents has considerable advantages to describe in detecting new bacteria, while also as described earlier acknowledging the pitfalls associated with it. Our results clearly demonstrate that bacterial population in sputum from CF patients was complex. In the following results chapters, we conducted experiments to investigate if the common anaerobes seen in CF were pathogenic as their pathophysiology and implications remain unclear.

Results:

Pathogenicity of Anaerobic Proteases

Chapter 4

4.1 Introduction

Anaerobic bacteria have always been difficult to grow under routine culture techniques. However, with the advancement of new molecular techniques, there is evidence, that there is a high prevalence rate of anaerobic bacteria in CF BAL and sputum (Tunney, Field et al. 2008). Currently, no data exists demonstrating the clinical impact of any of the anaerobic genra found in the CF lung. However, there has been some data that a presence of *P. aeruginosa* may confer the likelihood of having an increased number of anaerobes (Duan K 2003

, Tunney, Field et al. 2008). Data from Ulrich and colleagues (Ulrich, Beer et al. 2010) showed that the majority of patients with CF (94.1%) produced antibodies against two immunoreactive antigens of *P. intermedia*. Culture from *P. intermedia* cells, were more cytotoxic to respiratory epithelial cells in vitro and inflammatory in mouse lungs than respective fluids from anaerobically grown *P.aeruginosa*.

A high protease burden in the lung is characteristic of CF and causes increased levels of inflammation in the CF lung (Greene and McElvaney 2009). *P. aeruginosa* secretes pseudomonas elastase and alkaline elastase and has been shown to disrupt the tight junctions of the respiratory epithelium (Suter 1994). However, in this chapter we investigated if proteases are produced by anaerobic bacteria and if these proteases can inhibit antimicrobial peptides and cleave innate human defensins.

4.1.1 Measuring protease activity - Sensolyte Assay: Measurement of proteolytic activity in *P. melaninogenica* supernatants

A high pulmonary protease burden is characteristic of CF and the resulting dysregulation of the protease/anti-protease balance has serious consequences for inflammation in the CF lung (Geraghty, Rogan et al. 2007). Previous work has shown that many aerobic bacteria including *P. aeruginosa* are responsible for creating this heavy protease burden (Döring, Obernesser et al. 1983). This chapter focuses on, one of the most common anaerobes found in the lung, *P. melaninogenica,* and if it's proteases can contribute to the pathogenicity of the CF airway. *P.melaninogenica* was grown in BAM media and LB broth under anaerobic growth conditions in an anaerobic chamber. Supernatants were collected, centrifuged for 5 minutes at 6000 g and stored at days 3, 4,5,6,7. The *P. melaninogenica* used was cell free for all experiments. The proteolytic activity was measured by using the sensolyte assay which detects generic protease activities (e.g. trypsin, chymotrypsin, thermolysin, proteinase K, protease XIV, and elastase) using casein which is heavily labelled with 5(6)-TAMRA, a pH insensitive red flurophone. The highest fluorescent activity was found in samples grown on days 4-6 in LB and BAM media. This high fluorescent signal represents highest amount of proteolytic burden present suggesting that protease peaks at these intervals.

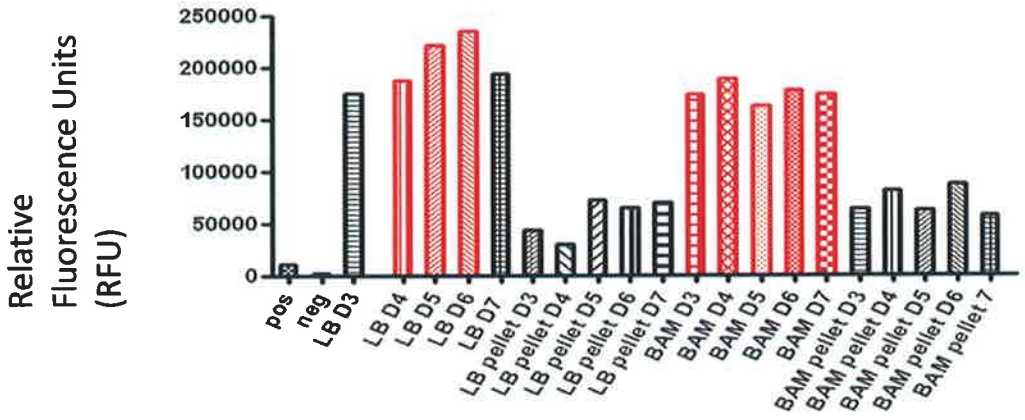


Figure 4.1: Measurement of proteolytic activity in P. melaninogenica.
The protease activity from BAM, LB broth grown neat and *P. melaninogenica* grown in various media from day 3 to day 7 was measured. These bacterial supernatants were extracted daily on Day 3 and Day 7 having been grown either in BAM or LB broth. Prepared protease substrate solution as described in section 2.14 was added. Control samples-positive trypsin and negative deionised water. Bacterial pellets from the bacteria on Day 3-7 were also used. Protease solution contained a flurophone, fluo-4am, that yields a red fluorescence, which can be continuously monitored at excitation/emission= 546nm/575nm. The increase in fluorescence intensity is directly proportional to protease activity (n=4).The red bars represent data obtained with bacterial supernatants and black bars represent data with bacterial suspension/pellet.

Proteolytic activity was highest in samples on days 4-6 in both LB and BAM broth (Figure 4.1).This indicates that proteases produced by the bacteria, *P. melaninogenica* were peaking at these intervals and in order to characterise these proteases, supernatants were subsequently collected at day 4.The absorbance at 546nm reflects the proteolytic activity of the bacteria. This experiment was repeated n=4 and all subsequent experiments used day 4 as the cut off for highest protease burden.

4.1.2 Zymography of bacterial supernatants, *P. melaninogenica*

The presence of proteases was also confirmed by using zymography.Novex zymogram gels (Life technology) are useful in detecting and characterizing proteases, which catalyse casein or gelatin as a substrate. Zymography is a technique using electrophoresis; it can be used to measure enzymatic activity.

P. melaninogenica was incubated in both BAM anaerobic media and LB media from day 3 until day 7 and grown in an anaerobic chamber. The *P. melaninogenica* supernatants were prepared in a non-reducing loading buffer, underwent electrophoresis and were then stained with Coomassie Blue. This data also confirmed that proteolytic activity is most active on day 4 and day 5 of *P. melaninogenica* grown in BAM media. The proteolytic activity is highest in bacteria on day 4 and day 5 in BAM broth. The supernatants from day 4 were therefore used in further experiments in order to characterise the protease activity (Figure 4.2).

The protease activity for zymography (figure 4.2) is only seen on day 4 and 5. This reflects the fact that zymography is important at degrading gelatin specifically gelatinase A and B. Sensolyte assay is a different technique used to examine caseinolytic and gelatinolytic activity more broadly and this maybe a reason why enzyme activity is seen in other days in this experiment.

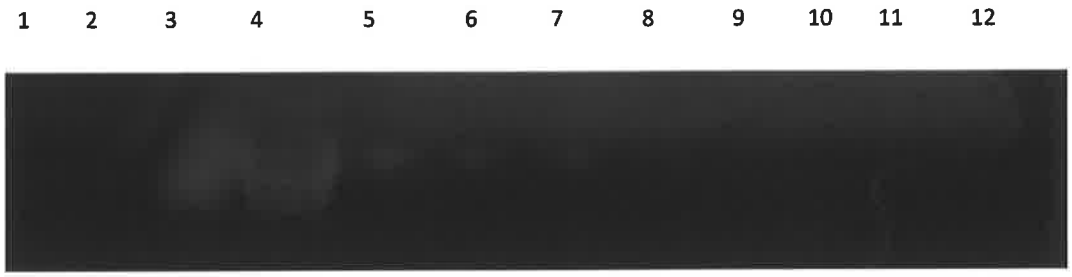


Figure 4.2 : Zymography of proteins after gel electrophoresis.
Following separation on a Novex® Zymogram Gelatin Gel (10%), proteins were renatured using Novex® Zymography Renaturing Buffer to allow substrate cleavage. Coomassie Blue staining of gel results in clear area where substrate was digested by protease. Lane 1: EMPTY LANE. Lanes 2-7: bacterial supernatants of *P. melaninogenica* between on day 3, 4, 5, 6, 7, 10 in BAM media respectively. Lanes 8-10: bacterial supernatants of *P. melaninogenica* on day 3, 4, 5, 6, 7 in LB media. Zymography demonstrates that P. melaninogenica supernatants in BAM media have proteolytic on Day 4 and Day 5 (lanes 3 and 4). No activity is displayed in the non-anaerobic media LB broth.

4.2.1 Anaerobic bacterial proteases can cleave antimicrobial peptides

Proteases from *P. melaninogenica* may be destructive in the CF lung (Zemanick, Harris et al. 2013).In the following set of experiments, two of the most common antimicrobial peptides in the lung, LL-37 and lactoferrin were incubated with bacterial proteases from *P. melaninogenica* to see what effects these proteases had on these naturally occurring peptides against the bacteria.

4.2.2 LL-37

LL-37 has strong anti-microbial properties (Turner, Cho et al. 1998). Other innate antimicrobial molecules such as defensins and SLPI, have been shown to be inactivated in the CF lung, because they are susceptible to degradation of the bacterial proteases (Taggart, Lowe et al. 2001, Taggart, Greene et al. 2003). In these experiments, proteases from *P. melaninogenica* were incubated for 4 days in the anaerobic chamber and were further incubated for 48 hours with LL37. LL 37 was found to be degraded by western blot analysis. Degradation products were also formed, thus indicating that *P. melaninogenica,* after 48 hours, loses LL-37 anti-microbial properties. LL-37 also has a role as an anti-inflammatory molecule in the lung (Brown, Poon et al. 2011) (Jönsson and Nilsson 2012).The formation of degradation products could also increase the IL-8 response and worsen inflammation (Zuyderduyn, Ninaber et al. 2006).

LL-37 was incubated with *P.melaninogenica* grown in BAM broth for 24, 48 and 72 hours. *P. melaninogenica* grown for 4 and 5 days was incubated in the presence of 8.5×10^{-7} M recombinant LL-37 for 24, 48 and 72 hour intervals at 37 °C. Samples were electrophoresed by reducing SDS-PAGE followed by western blot analysis with a LL-37 antibody. Degradation products were formed after 48 hours incubation on both day 4 and day 5 of the bacteria incubation (Figure 4.3).

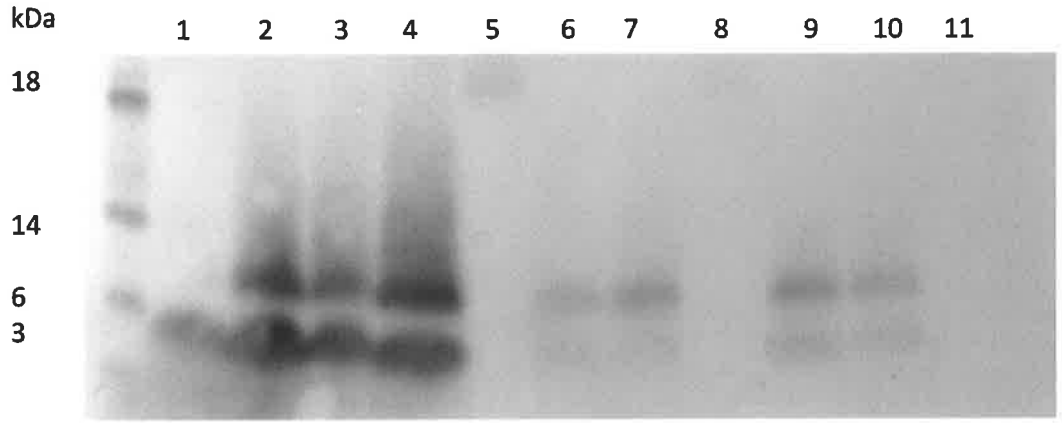


Figure 4.3: Incubation of LL-37 with P.melaninogenica grown in BAM broth for 24, 48 and 72 hours.
P. melaninogenica grown for 4 and 5 days was incubated in the presence of 8.5×10^{-7} M recombinant LL-37 for 24, 48 and 72 hour intervals at 37 °C. Samples were electrophoresed by reducing SDS-PAGE followed by western blot analysis using a LL-37 antibody. Lane 1: LL-37 standard. Lane 2-4: LL-37 in BAM broth for 24, 48, 72 hours respectively. Lane 5: EMPTY LANE. Lane 6-7: LL-37 incubated for 48 and 72 hours respectively in 4 day *P. melaninogenica* cultured medium. Lane 9-10: LL-37 incubated for 48 and 72 hours respectively in 5 day *P. melaninogenica* cultured medium. Lane 11: EMPTY LANE. The molecular weight of LL-37 is 3-4 kDa. Degradation products are seen after 48 and 72 hours respectively on both days

4.2.3 Lactoferrin

Lactoferrin is a 80kDa iron binding glycoprotein and has antimicrobial activity and forms part of the innate defence mechanism by binding to iron and sequestering it (Arnold, Cole et al. 1977, Ganz 2002). In the following set of experiments, we investigated if lactoferrin was cleaved by proteases from *P. melaninogenica*. Lactoferrin was incubated with *P.melaninogenica* grown in BAM broth for 24, 48 and 72 hours, in the same manner as LL-37. P. melaninogenica, grown for 4 and 5 days was incubated in the presence of 8.5×10^{-7} M recombinant lactoferrin for 24 h, 48h and 72h at 37 °C. Samples were electrophoresed by reducing SDS-PAGE followed by western blot analysis using a lactoferrin antibody. Degradation products were formed following 72 hours incubation of both day 4 and day 5 of the bacteria. Formation of degradation products suggests that lactoferrin can lose its anti-microbial activity after 72 hours of incubation with *P. melaninogenica*. This data also suggests that *P.melaninogenica* reduces the naturally occurring anti-microbial activity in the anaerobic broth (Figure 4.4).

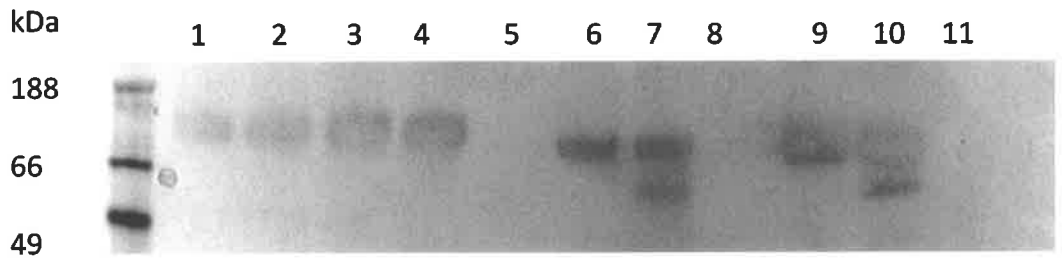


Figure 4.4 Western blot analysis of Lactoferrin presence in supernatants. Lactoferrin was incubated with *P.melaninogenica* grown in BAM broth for 24, 48 and 72 hours, this is the positive control. *P. melaninogenica,* grown for 4 and 5 days was incubated in the presence of 8.5×10^{-7} M recombinant lactoferrin at 24, 48 and 72 hour intervals at 37 °C. Samples were electrophoresed by reducing SDS-PAGE followed by Western blot analysis using a lactoferrin antibody. Degradation products were formed on 72 hours incubation of both days 4 and day 5 of the bacteria. All experiments done n=3. Lane 1: lactoferrin standard. Lanes 2-4: lactoferrin in BAM broth for 24, 48, 72 hours respectively. Lane 5: EMPTY LANE. Lane 6-7: lactoferrin incubated for 48 or 72 hours respectively in 4 day *P. melaninogenica* cultured medium. Lane 8: EMPTY LANE. Lane 9-10: lactoferrin incubated for 48 or 72 hours respectively in 5 day *P. melaninogenica* cultured medium. Lane 11: EMPTY LANE.

4.2.4 AAT

AAT is a 52kDa serine protease inhibitor often found in the lung, synthesised in the liver. It is also expressed in other types of cells including neutrophils, monocytes, macrophages, and alveolar macrophages (Grimstein, Choi et al. 2011). The normal serum level of AAT is 1-2mg/ml. It is an innate anti-protease that inhibits neutrophil protease and proteinase 3.

4.3.1 Bacterial proteases cleave AAT, an innate anti-protease

Proteases from *P.melaninogenica* were cultured in anaerobic broth, BAM for 4 days and incubated with 3µg/dl of AAT. It showed by western blot analysis that after 24 hours, AAT was cleaved, degradation products and cleavage complexes were formed. AAT activity was reduced when incubated with anaerobic bacteria proteases. Bacterial proteases from *P. melaninogenica* cleaved and inactivated AAT. AAT was incubated with *P.melaninogenica* grown in BAM broth for 24, 48 and 72 hours.

P. melaninogenica grown for 4 days was incubated in the presence of 3.1 × 10^{-6} M recombinant AAT at 24, 48 and 72 hour intervals at 37 °C. Samples were electrophoresed by reducing SDS-PAGE, followed by western blot analysis using an AAT antibody. Degradation products were formed following 72 hours incubation of the bacteria. AAT complexes formed after 72 hours incubation of recombinant AAT with *P. melaninogenica.* The activity of AAT as a serine protease inhibitor was inactivated by proteases produced by *P. melaninogenica.* Degradation products were formed after 24 hours of incubation with the bacteria (Figure 4.5).

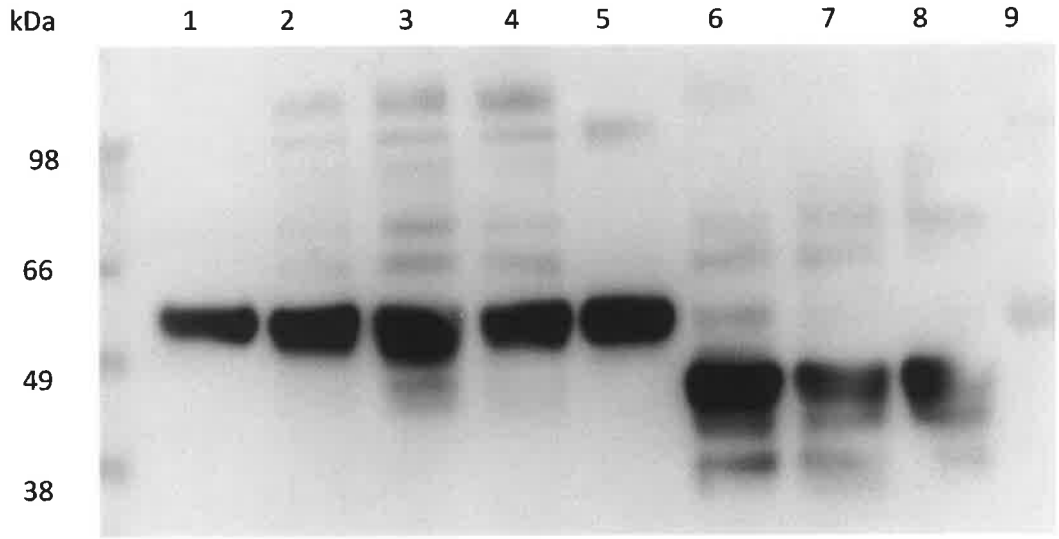


Figure 4.5: Western Blot analysis of AAT in bacterial supernatants.
P. melaninogenica grown for 4 days was incubated in the presence of 3.1×10^{-6}M recombinant AAT for 24, 48 and 72 hours respectively at 37 °C. Samples were electrophoresed by reducing SDS-PAGE followed by western blot analysis using AAT antibody. All experiments done n=3. Lane 1: AAT standard. Lane 2-4: AAT in BAM broth for 24, 48, 72 hours respectively. Lanes 5-8: AAT incubated for 0, 24, 48, 72 hours respectively in 4 day *P. melaninogenica* cultured medium. Lane 9: EMPTY LANE. AAT is degraded by 24 hours

4.3.2 Identification of the Protease(s) Involved in the Cleavage of AAT of Prevotella melaninogenica in anaerobic BAM media

To identify the protease(s) involved in the cleavage of AAT of *Prevotella melaninogenica* in anaerobic BAM media, different protease inhibitors were preincubated with *P.melaninogenica* samples prior to adding recombinant AAT (Figure 4.6). Following 1 hour incubation at 21 °C, samples were analysed by western blot under reducing or non-reducing conditions using an anti-AAT antibody. First, the use of nonspecific protease inhibitors targeting each protease family (serine, cysteine, acidic proteases, and metalloproteases) allowed identification of the family of the *P.melaninogenica* protease(s) involved in the

cleavage of AAT. Pefabloc, a nonspecific serine protease inhibitor, degrades AAT cleavage in *P.melaninogenica*, whereas neither E-64 (cysteine protease inhibitor), pepstatin A (acidic protease inhibitor), nor ACT (1- anti-chymotrypsin, a chymotrypsin inhibitor) and aprotonin (trypsin inhibitor) had any effect. In addition to pefabloc, other metalloproteinase inhibitors (EDTA, GM6001, phosphoramidon) and a serine protease inhibitor, PMSF were used to identify more precisely the serine protease involved in the cleavage of AAT.

A panel of protease inhibitors was used to identify the family of proteases produced by *P. melaninogenica* and are responsible for cleaving AAT (Figure 4.6). PI (protease inhibitor cocktail) Phenylmethanesulfonyl fluoride, (PMSF) (serine protease inhibitor) at a concentration of 10mM, E-64 (cysteine protease inhibitor) at 0.4mM, EDTA (metalloprotease inhibitor) 13mM, pefabloc (serine protease inhibitor) at 10mM; PEP A (cysteine peptidase) at 0.4mM, ACT (chymotrypsin inhibitor) at 1µm and Aprotonin (trypsin inhibitor) at 13µm. There is degradation and cleavage of AAT seen in lanes 6-12, by the various protease inhibitors suggesting that the protease involved is either a serine or a metalloprotease.

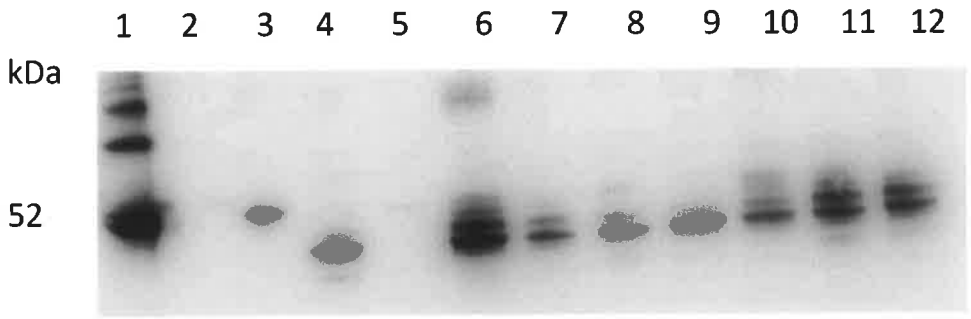


Figure 4.6: Effect of various protease inhibitors on the cleavage of recombinant AAT in *P. melaninogenica* anaerobically grown in BAM media.
P. melaninogenica grown in BAM anaerobic media on day 4 was preincubated for 1 h at 37 °C with various protease inhibitors. Recombinant AAT (3.1×10^{-6} M final concentration) was added to the samples and incubated for 24 h at 37 °C. Samples were electrophoresed on a 17.5% Tricine/SDS-PAGE under reducing conditions and analyzed by Western blot using an anti-AAT antibody. Western blot analysis from supernatants of *P. melaninogenica* and a panel of protease inhibitors, 3µg of AAT protein is added to each sample. Lane1: AAT standard. Lane 2: EMPTY LANE. Lane 3: protease inhibitor. Lane 4: BAM media. Lane 5: *P. melaninogenica* . Lane 6: PMSF. Lane 7: E-64. Lane 8: pepstatin A. Lane 9: pefabloc. Lane 10: EDTA. Lane 11: antichymotrypsin. Lane 12: Aprotonin. Degradation is seen in lanes 6-12 suggesting that the protease involved is both a serine and metalloprotease.

4.3.3 Other antiproteases-Elafin and SLPI

Elafin is a cationic 6kDa non-glycosylated serine protease inhibitor (Zani, Nobar et al. 2004).Elafin is a powerful inhibitor of neutrophil elastase and proteinase 3 (Ying and Simon 1993, Zani, Nobar et al. 2004).

SLPI is a 11.7 kDa cationic, non-glycosylated serine proteinase inhibitor, that is present on mucosal surfaces on many cells including neutrophils and macrophages (Weldon, McGarry et al. 2007). It inhibits a variety of proteinases, including NE, cathepsin G, trypsin, chymotrypsin, chymase and tryptase (Doumas, Kolokotronis et al. 2005). In the following set of experiments, the

antibacterial properties of SLPI and elafin on *Prevotella* species and *P. aeruginosa* grown anaerobically were investigated.

4.3.4 Cleavage of host protective molecules.

The degradation of secretory leukoprotease inhibitor (SLPI), alpha 1 antiprotease (AAT) and elafin by *P. aeruginosa*, *P. melaninogenica*, *P. nigrescens* and combination of both bacteria were evaluated. Proteases from *P. melaninogenica, P. aeruginosa* and a combination of both bacteria were cultured in anaerobic broth BAM for 4 days and incubated with 1µg/ml of SLPI and elafin respectively. Western blot analysis after18 hours of incubation with SLPI and elafin showed that these anti-proteases were cleaved, and degradation products and cleavage complexes were formed.SLPI is cleaved by bacterial proteases from both *P. melaninogenica* and *P. aeruginosa* following 18 hours incubation.

Elafin also formed cleavage and degradation products, when incubated in combination with *P. melaninogenica* and *P. aeruginosa,* grown for 4 days individually and combined when incubated in the presence of 2.4×10^{-7} M recombinant SLPI and 1.9×10^{-7} M respectively for 18 hours at 37 °C. Western blot analysis using a SLPI antibody as described previously and similarily an elafin antibody was performed. Degradation products were formed after18 hours incubation of day 4 of the bacteria (Figure 4.7).

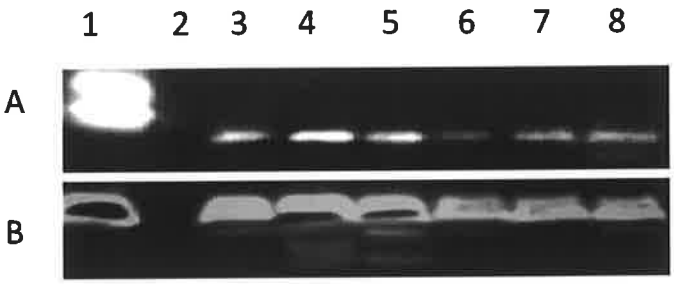


Figure 4.7: Cleavage of Elafin and SLPI.
Lane 1: SLPI standard forming a dimer on A, elafin standard on B. Lane 2: EMPTY LANE. Lane 3: *P. aeruginosa* and SLPI or elafin respectively. Lane 4: *P. melaninogenica* and SLPI or elafin respectively. Lane 5: Combination of *P. melaninogenica* and *P. aeruginosa* and SLPI or elafin respectively. Lane 6: *P. aeruginosa* different species and SLPI or elafin respectively. Lane 7:*P. nigrescens* and SLPI or elafin respectively. Lane 8: Combination of *P. aeruginosa* and *P. nigrescens* and SLPI or elafin respectively. Degradation products formed in Lane 8 with SLPI and Lane 4, 5, 8 with elafin.

P. melaninogenica was chosen as the bacteria to investigate further in our experiments, as this was the most prevalent anaerobe found in CF sputum on culture and 16S RNA sequencing from the clinical recruitment. In vitro experiments have shown that proteases are produced from *P. melaninogenica* and these proteases have been shown to degrade antiproteases such as SLPI, elafin and AAT. The proteases involved in AAT degradation have been further characterized and are found mainly to be metalloproteinases and serine proteases.

Proteases produced from *P. melaninogenica* have also the ability to degrade LL-37 and lactoferrin which have important antimicrobial properties. This would suggest that *P. melaninogenica*, the most common anaerobe detected in our patient group, produces proteases, which are pathogenic by inhibiting the antiproteases and degrading antimicrobial proteins. *P. melaninogenica* would

appear from this data to be a new emerging pathogen in the CF lung.The degree of cleavage to which any of the innate human defences are cleaved, is determined by the inflammatory milleu and underlying disease process and it could suggest that anaerobes themselves are pathogenic by their own protease production, but that this could be augmented if they also are in a *Pseudomonas aeruginosa* rich environment as illustrated in the final experiment.

4.4 Discussion

P. melaninogenica produces proteases most abundantly on day 4 and 5 of culture.These proteases have been shown to inactivate LL-37 and lactoferrin in vitro.Lactoferrin has antimicrobial activity and forms part of the innate defence mechanism by binding to iron and sequestering it (Arnold, Cole et al. 1977, Ganz 2002). Lactoferrin can bind to cell membranes of gram negative bacteria leading to alterations in membrane permeability, which cause cell injury(Arnold, Russell et al. 1982). Lactoferrin can also bind to lipopolysaccharide (LPS) and enhance bacterial killing (Appelmelk, An et al. 1994). Cathepsins in CF sputum can cleave lactoferrin and consequently there is loss of antimicrobial and anti-biofilm activity (Rogan, Taggart et al. 2004). Lactoferrin has also been shown to undergo proteolytic cleavage in the lungs of CF patients infected with *P. aeruginosa* (Britigan, Hayek et al. 1993). Bacterial proteases from *P. melaninogenica*, in these experiments, have been shown to degrade lactoferrin and form degradation products after 72 hours. The loss of lactoferrin could have implications on the antimicrobial and anti-biofilm activity and could potentially lead to a more pro inflammatory environment.

LL-37 has strong anti-microbial properties (Turner, Cho et al. 1998). Other innate antimicrobial molecules such as defensins and SLPI, have been shown to be inactivated in the CF lung, because they are susceptible to degradation of the bacterial proteases (Taggart, Lowe et al. 2001, Taggart, Greene et al. 2003). Proteases from significant CF bacteria including *S. aureus* and *P.aeruginosa* have been shown to degrade LL-37 (Schmidtchen, Frick et al. 2002). Similarily, we have shown in these experiments that LL-37 is cleaved and degradation products are formed after 48 hours.

During inflammation, AAT level, as an acute phase reactant can increase 3-4 fold, suggesting an important role in responding to inflammation in the human body. It inhibits neutrophil elastase and proteinase 3 with high efficiency, as well as cathepsin G, thrombin, trypsin and chymotrypsin with lower efficiency (Macen , Upton et al. 1993). It also inhibits lipopolysaccharide (LPS)-stimulated release of TNF-α and interleukin (IL) -1β, and enhances the production of anti-inflammatory cytokine IL-10 (Dhami, Gilks et al. 2000). In our experiments, AAT has been shown to be cleaved after 48 and 72 hours by anaerobic proteases and degradation products are produced. The proteases involved in degradation are either a serine or a metalloproteinase. This suggests that the anti-inflammatory action could potentially be lost by AAT if it is degraded.

Elafin is a powerful inhibitor of neutrophil elastase and proteinase 3 (Ying and Simon 1993, Zani, Nobar et al. 2004). Elafin's main role is as an anti-protease to protect tissue against proteolytic destruction caused during inflammation (Sallenave, Shulmann et al. 1994, Sallenave, Si Tahar et al. 1997).

Elafin has also demonstrated anti-inflammatory and antibacterial properties particularly against *P. aeruginosa* and *S. aureus* (Simpson, Maxwell et al. 1999). These properties are potentially reduced if anaerobic proteases are produced as elafin has been shown to get cleaved.

SLPI inhibits a variety of proteinases, including NE, cathepsin G, trypsin, chymotrypsin, chymase and tryptase (Doumas, Kolokotronis et al. 2005). It also has antibacterial properties and because it is cationic, it can disrupt cell membranes on bacteria such as *P.aeruginosa, Staphylococcus aureus and Candida albicans* (Tomee, Koëter et al. 1998). *Prevotella* species and *P. aeruginosa* were both degraded by elafin and SLPI and degradation products were formed after 18 hours.

The proteases produced from *P. melaninogenica*, one of the most common anaerobes in the CF lung have now been shown to cleave antimicrobial peptides- lactoferrin and LL-37 and also to inhibit naturally occurring antiproteases AAT, SLPI and elafin.

Results: Short Chain Fatty Acids in Cystic Fibrosis

Chapter 5

5.1 Introduction

SCFAs are produced from anaerobic bacteria and their role is unknown. In this study we used the hollow fibre supported liquid membrane extraction coupled with gas chromatography to identify and quantify SCFAs present in supernatants of anaerobic bacteria. The method relies in selectively concentrating SCFAs within the lumen of the hollow fibre which are then analysed by gas chromatography. We wanted to show that the anaerobic bacteria that are present in the CF lung can produce SCFAs and that these are proinflammatory. We also found that bacteria have unique identifiers from each bacteria and a set amount of SCFAs are produced.

5.2.1 Linearity

We validated the hollow fibre method by determining its linear range for each of the SCFAs of interest. This ensures that the relationship between the analyte (SCFA) concentration and response (peak area) is linear, which then enables the determination of the enrichment factor and by doing so the quantitative determination of SCFAs. Table 5.1 shows the parameters for the linear regression and Figures 5.1 to 5.7 depict the linear relationship between the concentration of individual SCFA and response (peak area). The method is linear in the low micro-molar range for all SCFAs evaluated as evident from r^2 values. This enables their determination in samples that contain low amounts of SCFAs e.g. serum and plasma. Previous reports (Zhao, Liu et al. 2007) have shown that the serum values of acetic, propionic and butyric acid are 130-230μM, 11-15 μM and 9-17μM respectively.

SCFA	Linear range (µM)	Equation	r^2
Acetic acid	2 - 350	76.4x - 207.5	0.9872
Propionic acid	1 - 140	137.2x - 226.1	0.9916
i-Butyric acid	0.76 - 133	212.3x - 116.9	0.9927
Butyric acid	0.8 - 140	168.1x - 212.7	0.9926
2-Methylbutyric acid	0.4 - 70	201.6x - 184.2	0.9906
i-Valeric acid	0.4 - 70	229.3x - 78.9	0.9931
Valeric acid	1.44 - 252	145.7x - 977.3	0.9923

Table 5.1: Parameters for the regression equations (y = ax + b) of the SCFAs, where y is the donor concentration (mM) and x is the peak area counts.

The linear dynamic range is the concentration range of the detector output is linearly related to the solute concentration.

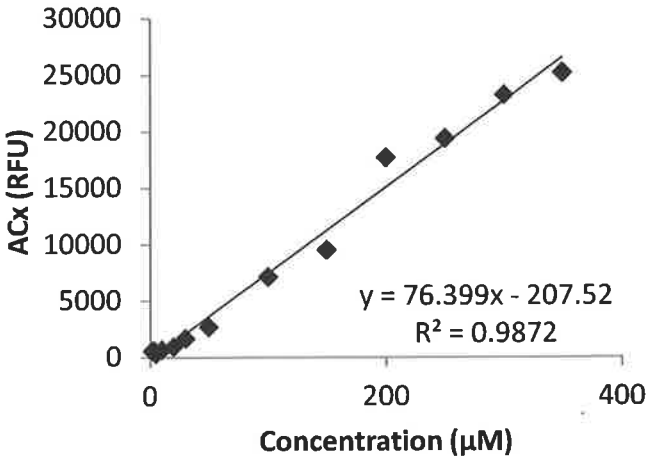


Figure 5.1: Graph depicting the linear relationship between the concentration of acetic acid and response (peak area).

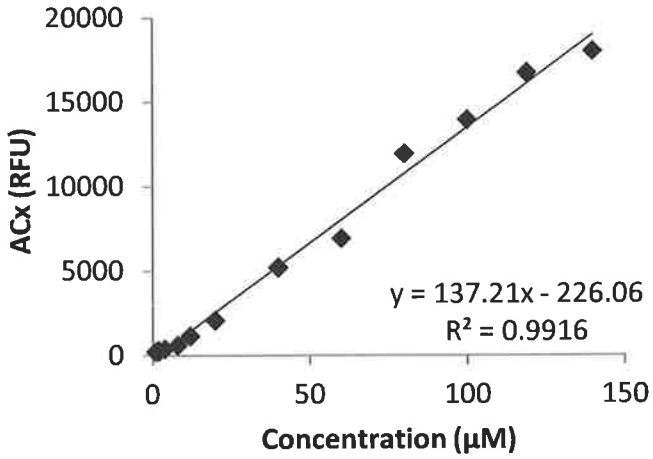


Figure 5.2: Graph depicting the linear relationship between the concentration of propionic acid and response (peak area).

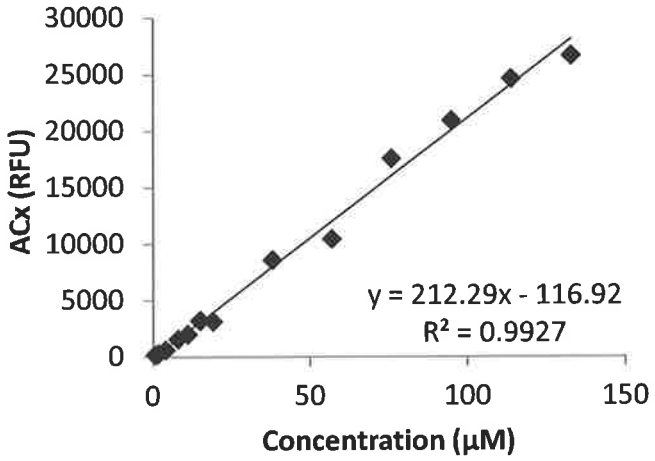


Figure 5.3: Graph depicting the linear relationship between the concentration of i-butyric acid and response (peak area).

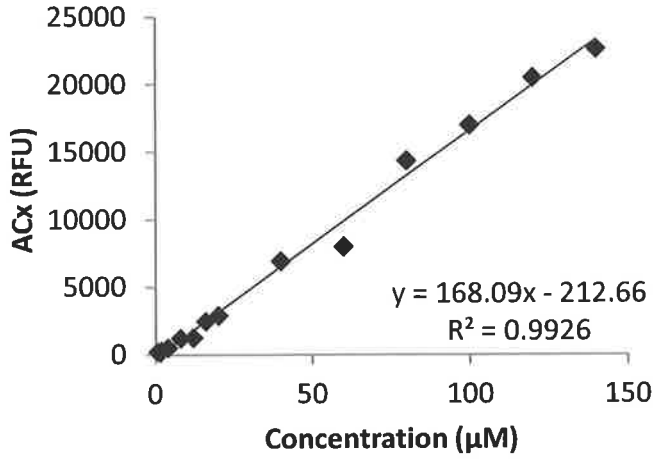


Figure 5.4: Graph depicting the linear relationship between the concentration of butyric acid and response (peak area).

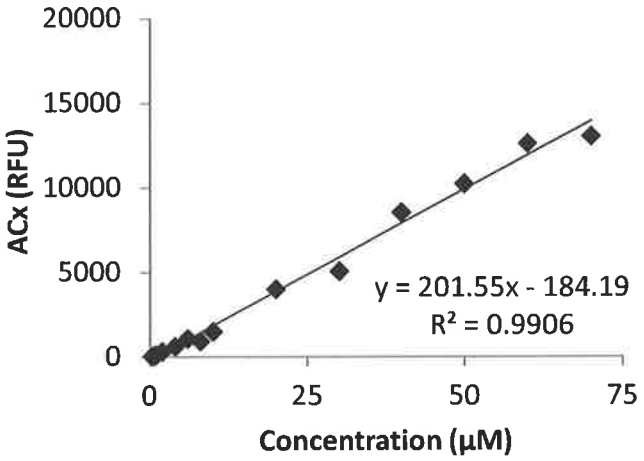


Figure 5.5: Graph depicting the linear relationship between the concentration of 2-methylbutyric acid and response (peak area).

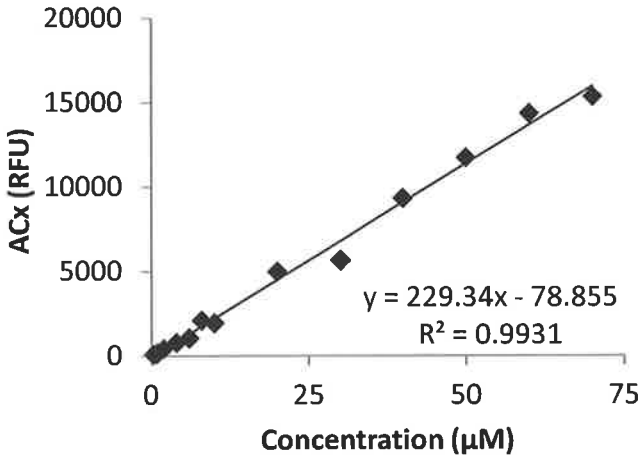


Figure 5.6: Graph depicting the linear relationship between the concentration of i-valeric acid and response (peak area).

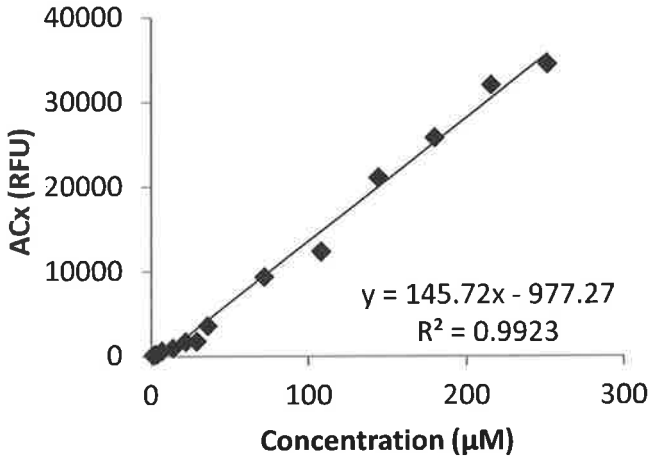


Figure 5.7: Graph depicting the linear relationship between the concentration of valeric acid and response (peak area).

5.2.2 Enrichment factor

The enrichment of the SCFAs occurs when the donor solution is acidified to pH 2 by addition of 2M HCl. All SCFAs have pKa from 4.5 - 4.9 this means in this pH all acids are protonated. In this state SCFAs are able to diffuse across from the bulk donor solution to the surface of the hollow fibre where they partition into the membrane liquid (Figure 5.8). At the luminal surface of the hollow fibre, however these SCFAs become ionised because of the high pH of 0.3M NaOH in this way becoming unable to get back into the membrane liquid. In addition, basic compounds that are in the donor solution are in their protonated form and cannot therefore be extracted. Thus, the hollow fibre allows selective and efficient extraction of SCFAs from various samples.

To determine the factor by which the SCFAs get enriched from donor to acceptor solution, different dilutions of SCFAs standard mixture were prepared

and analysed. By dividing the SCFAs concentrations determined in the acceptor solution with SCFAs concentrations in the donor solution, the enrichment factor was determined for each dilution. The average values are shown in Figure 5.9.

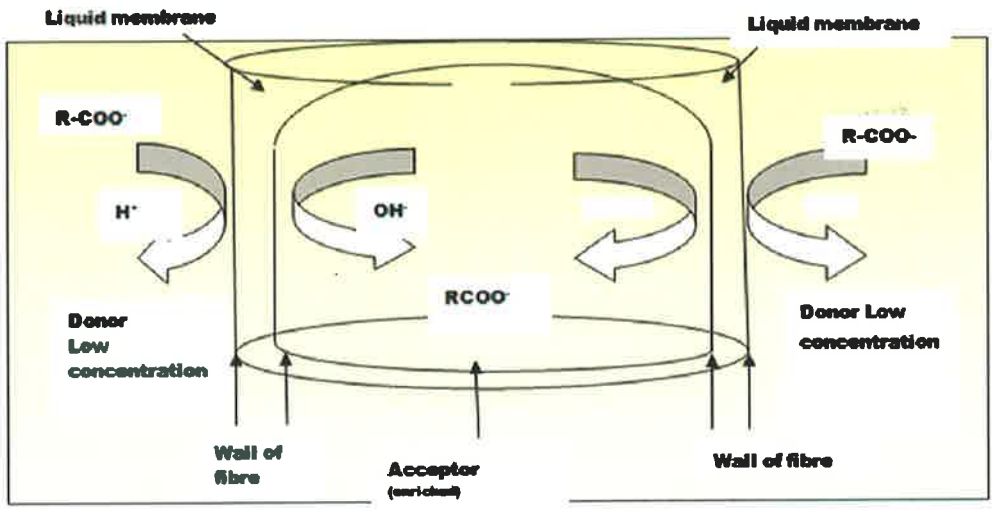


Figure 5.8: Extraction of SCFA with hollow fibre supported membrane adapted from Zhao et al.(Zhao, Liu et al. 2007)

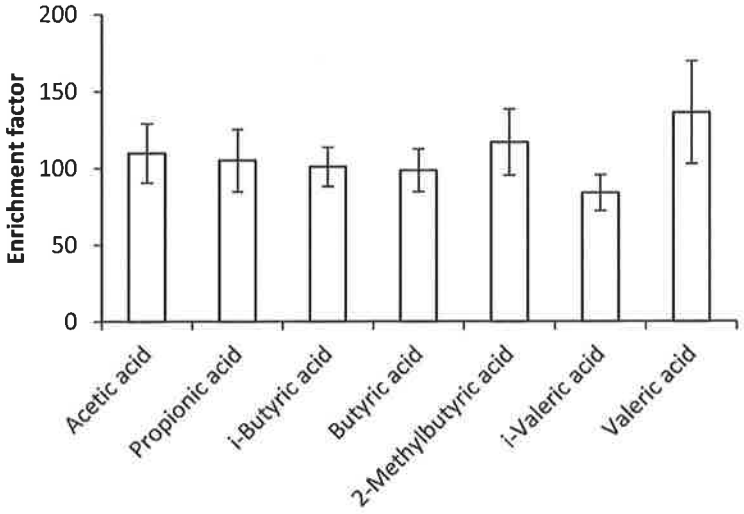


Figure 5.9: Enrichment factor (n=6)

5.3 Identification and quantification of SCFA produced by five key anaerobes identified in the lung

Five anaerobic bacteria were identified as the key and mostly likely pathogenic in the lung. These included *S. sanguinis, F. nucleatum, Actinomyces odontolyticus, P. melaninogenica,* and *V. parvula.* In this study we evaluated both; ATCC obtained strains as well as strains isolated from patient samples (BAL and sputum). In both cases, all evaluated bacteria secreted mM concentrations of SCFAs with acetic, propionic and butyric acid being one of the most abundant ones (Figures 5.10 -5.14). Furthermore, ATCC and patient isolated strains had identical SCFAs secretion profiles with similar amounts of secreted SCFAs.

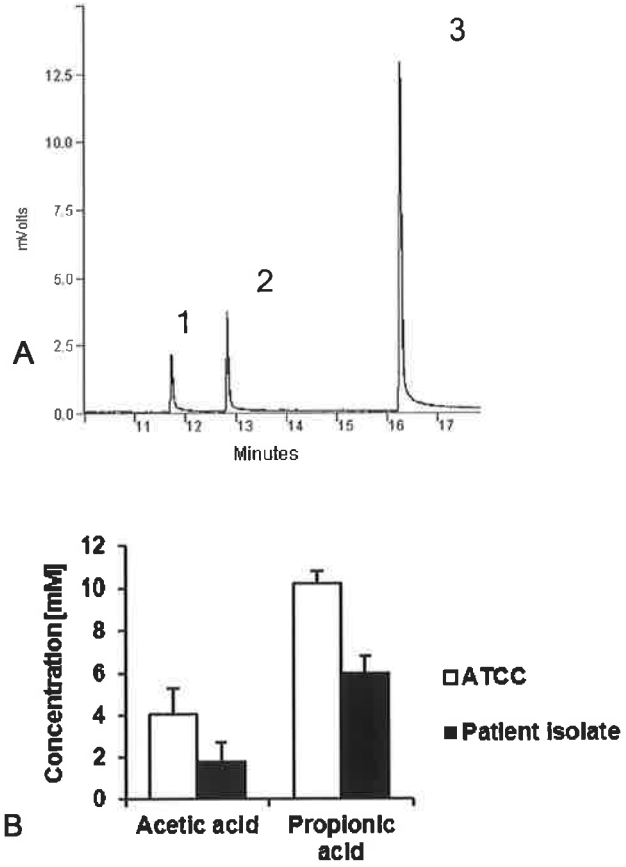


Figure 5.10: *Veillonella parvula* secretes mM concentrations of acetic and propionic acid.

A) A representative chromatogram of *Veillonella parvula* supernatant. Peak 1, 2 and 3 represent acetic, propionic and 2-ethylbutyric acid, respectively. B) Both ATCC obtained clones and clones isolated from patient samples secrete acetic and propionic acid. Values are presented as means ± SEM, n = 4.

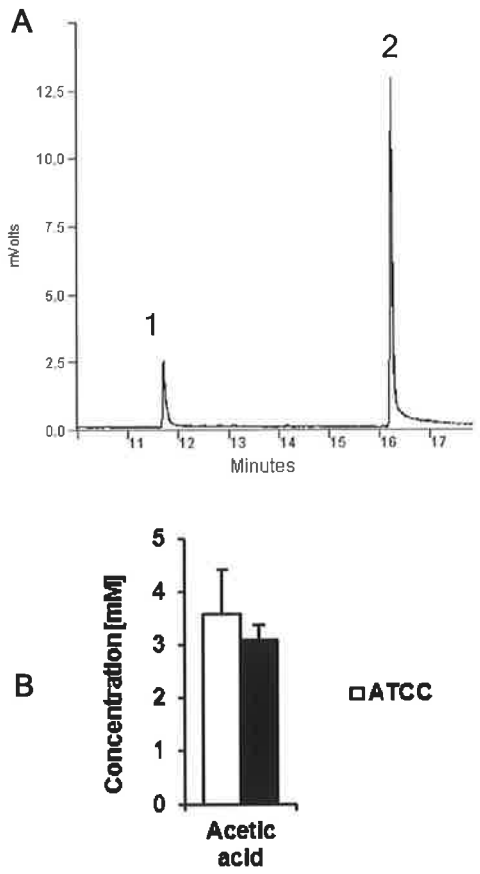


Figure 5.11: ***Streptococcus sanguinis*** **secretes mM concentrations of acetic acid.** A) A representative chromatogram of *S. sanguinis* supernatant. Peak 1 and 2 represent acetic and 2-ethylbutyric acid, respectively. B) Both ATCC obtained clones and clones isolated from patient samples secrete acetic acid. Values are presented as means ± SEM, n = 4.

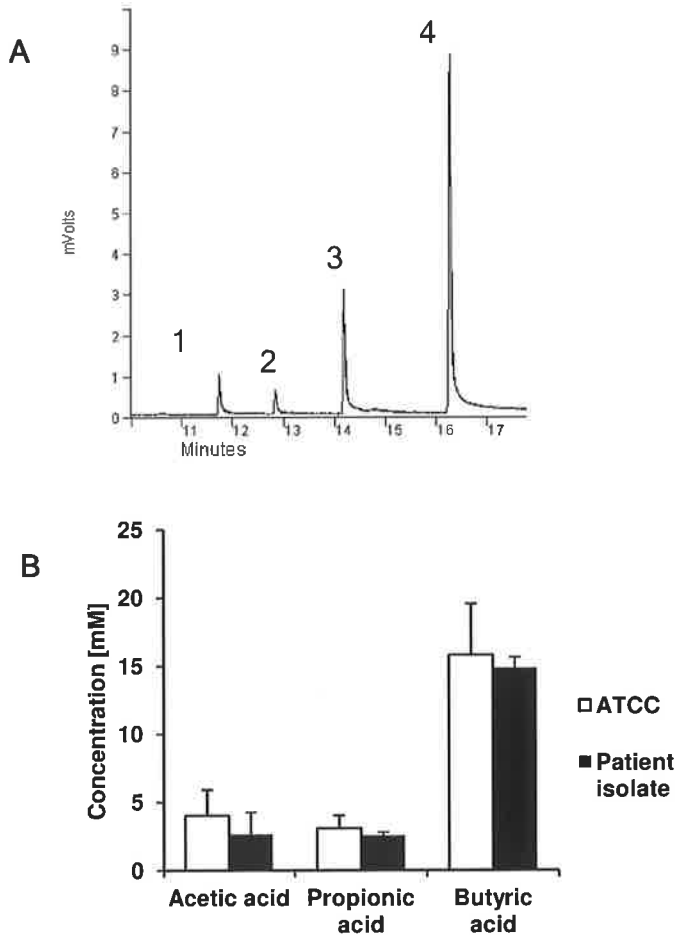


Figure 5.12: *Fusobacterium nucleatum* secretes mM concentrations of acetic, propionic and butyric acid.
A) A representative chromatogram of *F. nucleatum* supernatant. Peak 1, 2, 3 and 4 represent acetic, propionic, butyric and 2-ethylbutyric acid, respectively. B) Both ATCC obtained clones and clones isolated from patient samples secrete acetic, propionic and butyric acid. Values are presented as means ± SEM, n = 4.

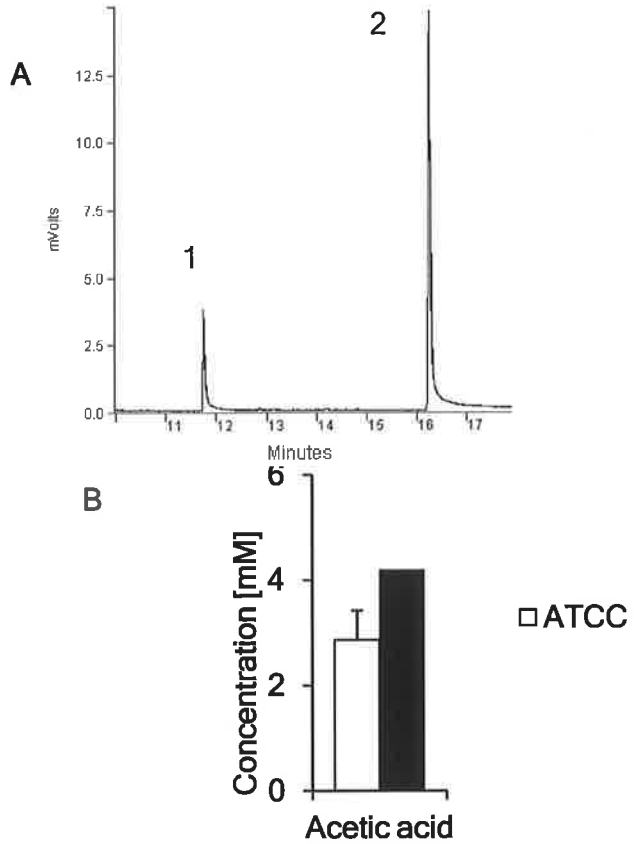


Figure 5.13: *Actinomyces odontolyticus* secretes mM concentrations of acetic acid. A) A representative chromatogram of *Actinomyces odontolyticus* supernatant. Peak 1 and 2 represent acetic and 2-ethylbutyric acid, respectively. B) Both ATCC obtained clones and clones isolated from patient samples secrete acetic acid. Values are presented as means ± SEM, n = 4.

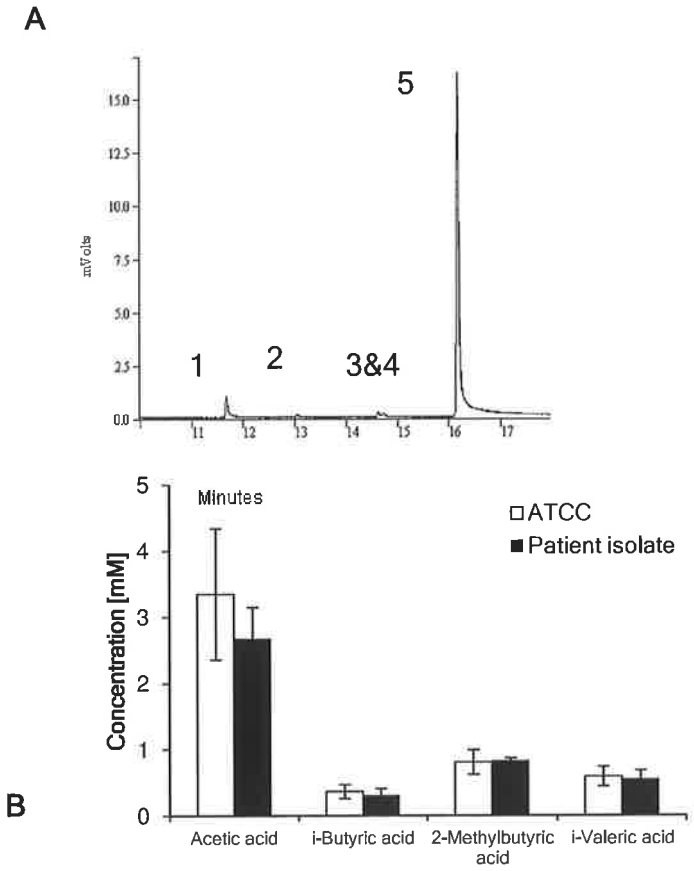


Figure 5.14: Prevotella *melaninogenica* secretes mM concentrations of acetic, *i*-butyric, 2-methylbutyric and *i*-valeric acid.
A) A representative chromatogram of *P. melaninogenica* supernatant. Peak 1, 2, 3, 4 and 5 represent acetic, *i*-butyric, 2-methylbutyric, *i*-valeric and 2-ethylbutyric acid, respectively. B) Both ATCC obtained clones and clones isolated from patient samples secrete acetic, *i*-butyric, 2-methylbutyric and *i*-valeric acid. Values are presented as means ± SEM, n = 4.

5.4 GPR 41 and GPR 43 expression in SCFA

5.4.1 GPR41 and GPR43 expression-GPR41 and 43 gene expression in healthy and CF lung epithelium

SCFAs are produced as by-products of bacterial fermentation in the gut. Recently, two orphan G-coupled receptors (GPR), GPR41 and GPR43 have recently been shown to act as receptors for SCFAs (Le Poul, Loison et al. 2003). Presence of GPR41 and GPR43 receptors has not yet been investigated in CF lung epithelium. First, we have chosen HBE and CFBE epithelial cell lines to evaluate the GPR41 and GPR43 gene expression by QRT-PCR. GPR41 and GPR43 were both present in the chosen cell lines; however, there was a significant increase in expression of GPR41 in CFBE cell line compared to HBE cell line ($p < 0.001$) (Figure 5.15 A, B).These results were further confirmed with agarose gel electrophoresis (Figure 5.15 C). Furthermore, we have also analysed GPR41 and GPR43 gene expression in bronchial brushings in healthy age matched controls and people with CF who were homozygous for the ΔF508 mutation. Results confirmed our data obtained with the two cell lines as GPR41 had increased expression in CF bronchial brushings compared to healthy controls (Figure 16). GPR43 on the other hand did not show significant difference between the cell lines or bronchial brushings (Figure 5.16 B and Figure 5.16 B). GPR 43 was found to be reduced in our CFBE cell lines (Fig 5.15 B).

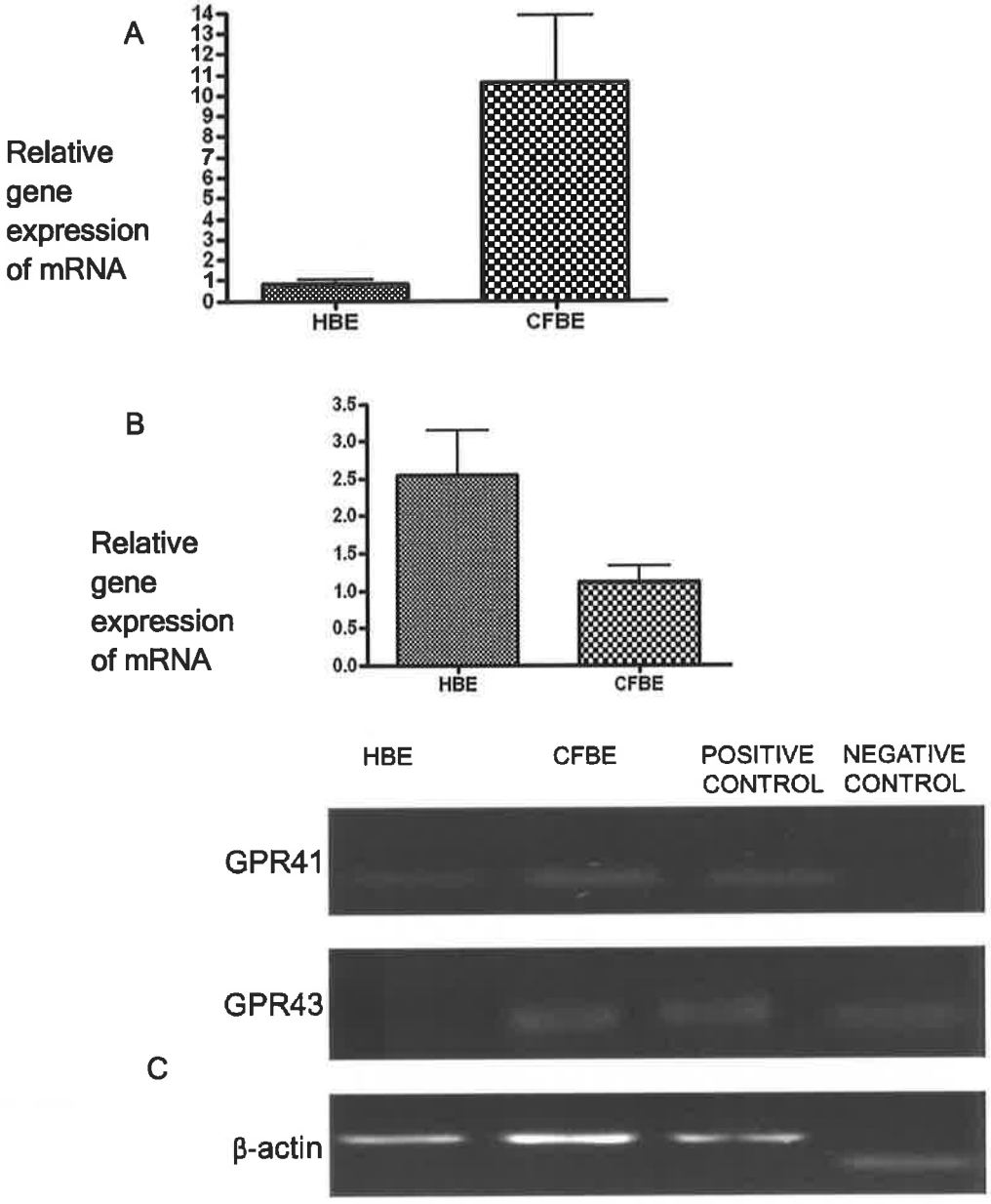


Figure 5.15: GPR41 (A) and GPR43 (B) mRNA levels in HBE and CFBE cell line as determined with QRT-PCR.
These results were further confirmed with 2% gel agarose analysis (C). All data are presented as means ± SEM, n = 6.

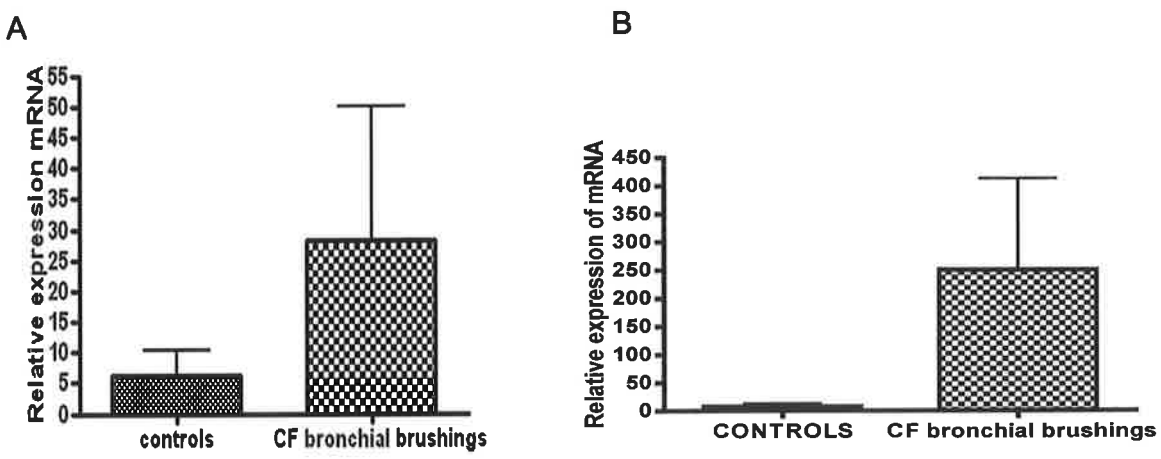


Figure 5.16: GPR41 (A) and GPR43 (B) mRNA levels in bronchial brushings from healthy controls and people with CF as determined with QRT-PCR. All data are presented as means ± SEM, n = 6

5.4.2 GPR41 and GPR43 protein expression

Next, we evaluated GPR41 and GPR43 protein expression in whole cell lysates and crude membrane fraction using Western blotting (Figure 5.17). The predicted molecular weight of GPR41 is 39-kDA (Brown, Goldsworthy et al. 2003). We looked at crude membrane fraction because we wanted additional proof that the receptor has increased expression on the cell surface as well. However, the main band for GPR41 was at higher molecular weight < 150 kDa in both whole cell lysates and crude membrane fraction suggesting oligomerisation of the receptor at the cell surface.This was previously suggested for certain GPR receptors (Salim, Fenton et al. 2002), however there are no studies reporting oligomerisation of the GPR41 receptor. In addition the band corresponding to GPR41 was much more pronounced in CFBE cell line compared to HBE cell line suggesting higher protein levels of GPR41 in CFBEs compared to HBEs. On the other hand, GPR43 has a predicted molecular weight of 43 kDa (Ge, Li et al.

2008), whereas the major band was apparent at approximately 50 kDa (Figure 5.17). No difference in band intensities was apparent with GPR43 suggesting no change in protein levels in CFBE cell line compared to HBE cell line.

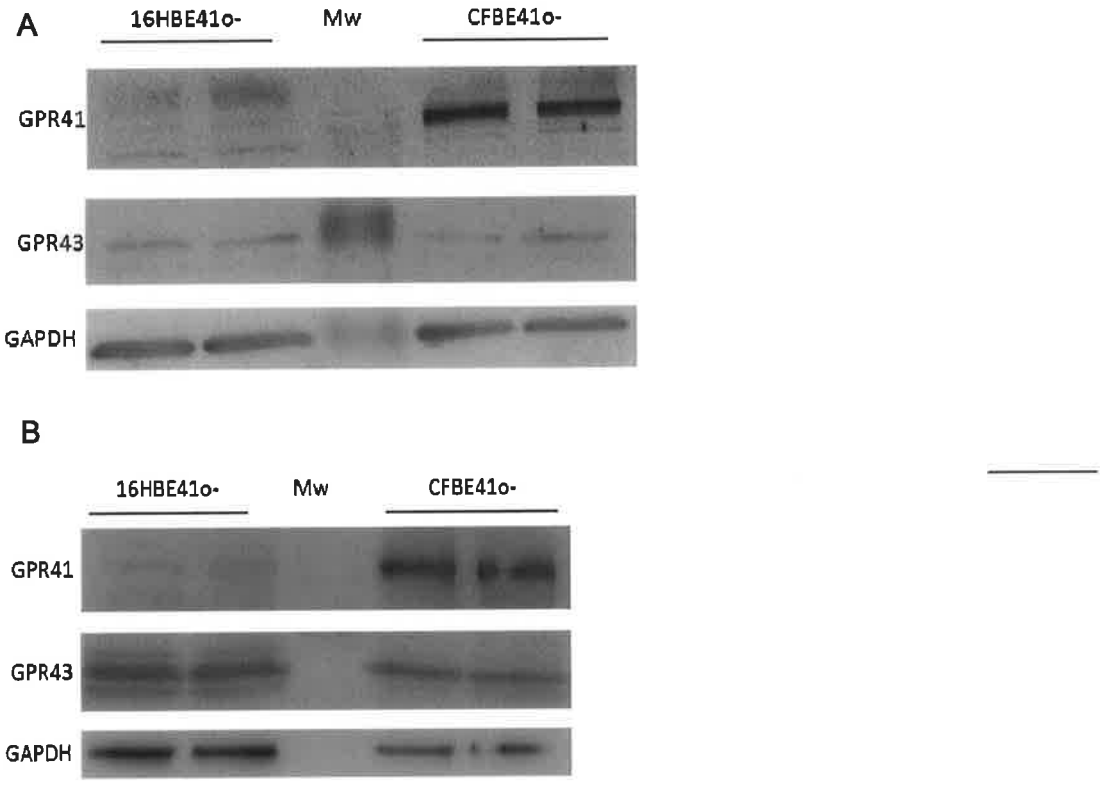


Figure 5.17: GPR41 and GPR43 protein expression in whole cell lysates
(A) Crude membrane fraction (B) Western blot analysis. GADPH was used as loading control. Data are presented as means ± SEM, n = 4.

5.4.3 Laser scanning cytometry

We investigated GPR41 and GPR43 cell surface expression using LSC. This method was chosen over flow cytometry to avoid artefacts caused by epithelial layer disruption induced by trypsinization. The expression of GPR41 receptor was significantly higher at the surface of CFBE cells compared to HBE cells (Figure 5.18A). The cell surface expression of GPR43 was not different in CFBE cells compared to HBE cells (Figure 5.18 B). These results confirm our previous data obtained by western blotting (see above).

A

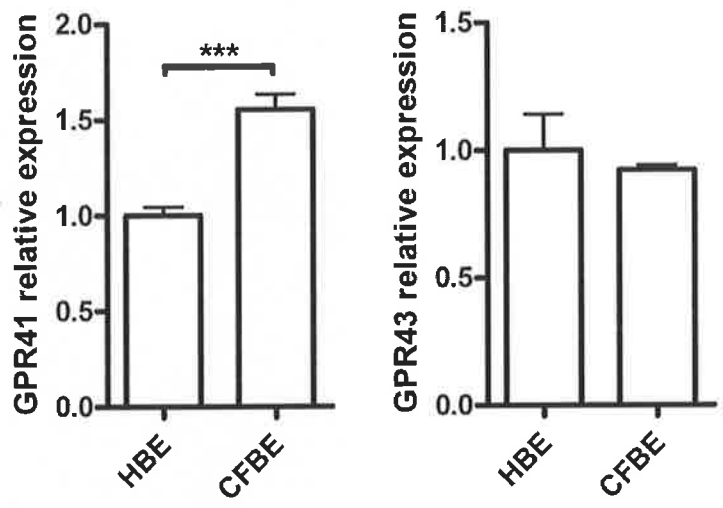


Figure 5.18: GPR41 (A) and GPR43 (B) cell surface expression as shown with laser scanning cytometry. Data are presented as mean ±, n = 3. ***p < 0.001.

5.5 Short chain fatty acids inflammatory response

5.5.1 Short chain fatty acids induce IL-8 production in CFBE cells

We investigated if SCFA can modulate the response in the CF lung. SCFA have been postulated to promote inflammatory responses at the site of bacterial infection. Therefore, we examined IL-8 production in CFBE and HBE cells on stimulation of various SCFA, including acetic, propionic, *i*-butyric, butyric, 2-methylbutyric and *i*-valeric acid (Figure 5.19). Pre- treatment of HBE and CFBE cells with increasing concentrations SCFAs led up to dose-dependent and a significant IL-8 release demonstrating that IL-8 is substantially increased when cells are also exposed to SCFAs. Propionic, butyric and *i*-valeric acid induced the most pronounced increase in IL-8 production whereas, acetic and *i*-butyric had a less pronounced effect and 2-methylbutyric displayed no IL-8 inducing effect.

Furthermore, in accordance with our previous results showing that CFBE cells have higher mRNA and protein levels of GPR41 compared to HBE cells, CFBE cells were more susceptible to SCFAs treatment and produced significantly more IL-8 than HBE cells.

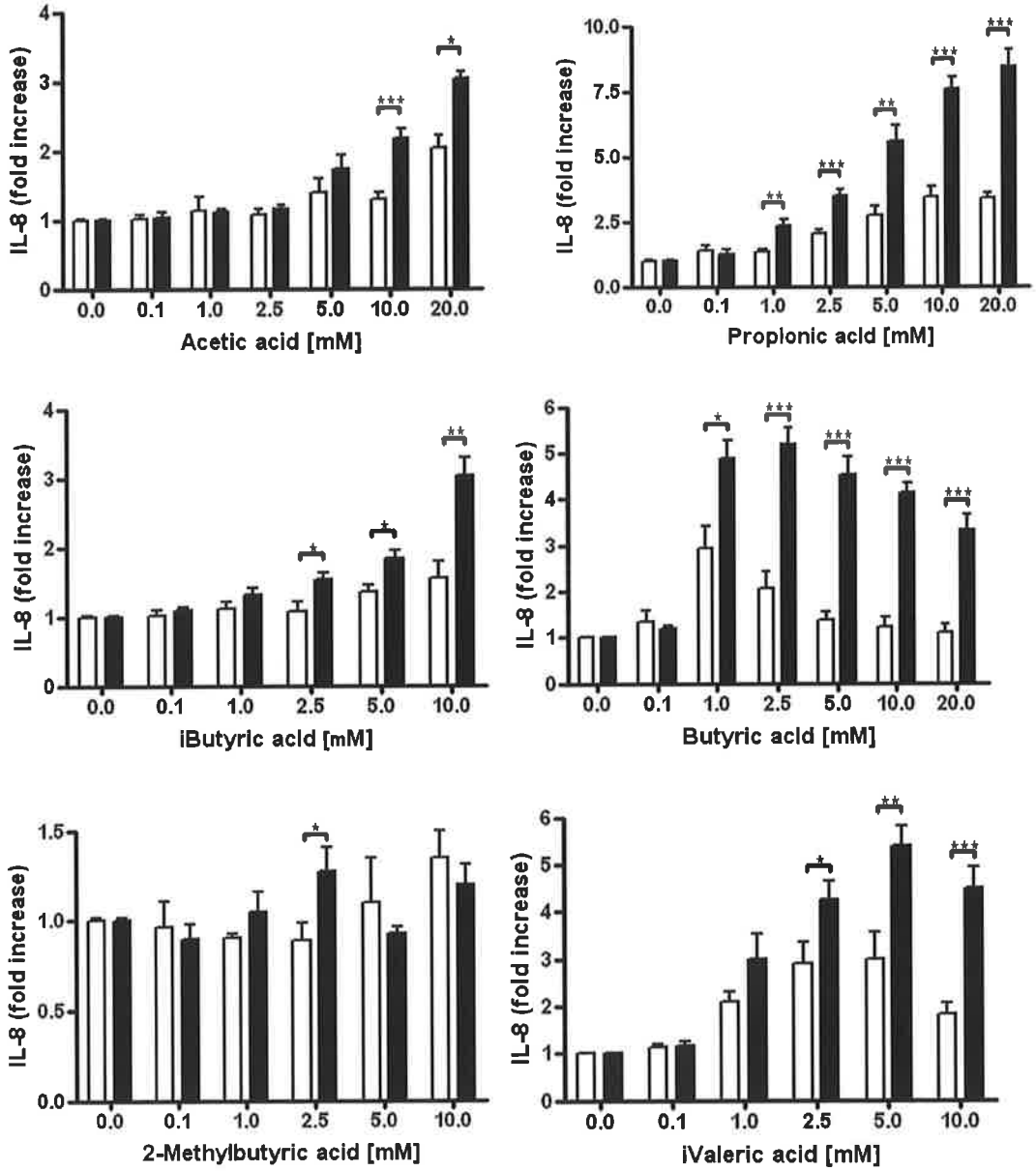


Figure 5.19: SCFA-induced IL-8 protein production in HBE cells and CFBE cells as shown with IL-8 ELISA.

1 x 10^5 cells were seeded in the wells of the 24-well plate and left overnight to adhere. Cells were then treated with increasing concentrations of SCFAs in MEM supplemented with 1% foetal bovine serum for 24 hours. Cell supernatants were collected and IL-8 levels were measured by ELISA. Data are presented as means ± SEM, n = 3. *p < 0.05, **p <0.01, ***, p<0.0001.

Of the selected five, three anaerobic strains (*F. nucleatum, V. parvula* and *P. melaninogenica*) simultaneously produced several different SCFAs. Therefore, we next sought out to evaluate whether the similar combination of these SCFAs would have a synergistic effect on IL-8 production on CFBE cells. All individual SCFAs when placed did have an increase in IL-8. However, when these SCFAs were combined together the SCFAs in combination did not induce a synergistic effect on IL-8 production (Figure 5.20). Further experiments would need to be performed to clarify the exact reason, however it is postulated that the SCFA may all work through the same receptor and this becomes saturated so the added effect isn't seen when the SCFA are combined.

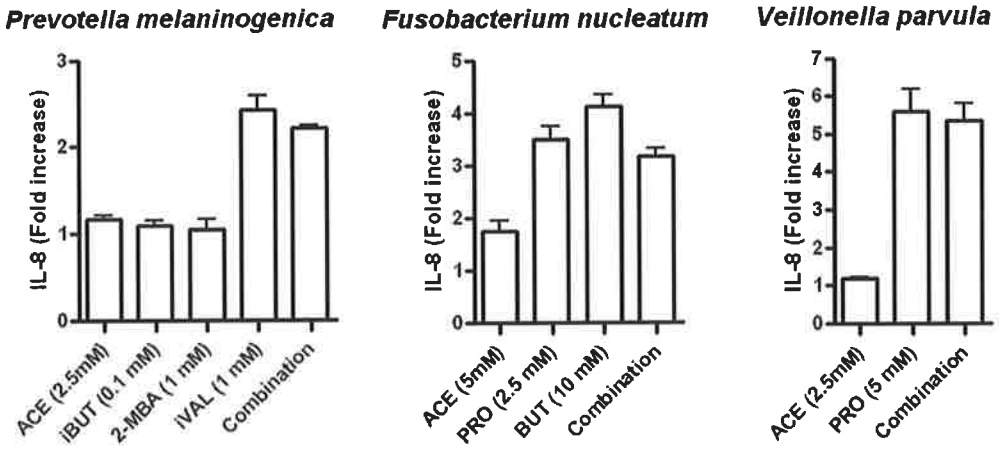


Figure 5.20: SCFAs combination treatment.

5.6 Discussion

Gas chromatography was used both as a quantification and identification tool of SCFA. This was done using the hollow fibre method in the experiments. It was used with the aim, primarily to try and elucidate if anaerobic bacteria in the lung could be identified by the types of SCFA identified in the CF lung. A unique set and quantification of SCFAs have been identified for the most common anaerobic bacteria found in the CF BAL and this could be used to fingerprint the bacteria in the future by identifying the SCFA involved.

GPR 41 and 43 receptors have been identified as the receptors for SCFA. There was increased GFR 41 receptor expression in both CFBE cells more than HBE cells and this was confirmed at both protein and m RNA expression. Laser scanning cytometry also confirmed increased expression of the receptor on CFBE cells.

Specific SCFAs are produced from different anaerobic bacteria and these increase IL-8 response when incubated with CFBE cells compared to controls. When these SCFA are accumulated together, there is a limited accumulative IL-8 response. This could be due to the saturation of GPR 41 receptor which mediates SCFA.

Discussion

Chapter 6

6.1 Discussion

It is believed that the CF airways are not infected at birth and that opportunistically pathogenic bacteria enter the lower airways from the environment. They are then able to establish a chronic presence in the airways due to the impaired innate immunity and chronic inflammatory response (Doring and Gulbins 2009).There is strong evidence that culture based methods only detect a small number of bacteria present in CF lungs (Tunney, Field et al. 2008).Over reliance of culture only methods also delays results and mis identifies the organisms present. In our study no anaerobes were detected by normal culture methods in both adult and children, however on subsequent sequencing 28% of the total microbiome was anaerobes. Previous work has shown, that comparing oral cavity flora and sputum from CF by 16S r RNA sequencing, sputum samples were not found to be contaminated with oral flora and these were used in our children over 12 and adult population in our study (Rogers, Carroll et al. 2006). 62% (28/45) of PA positive adult patients at both stable and exacerbation points grew anaerobes and the most common grown *P. melaninogenica, P. nigrescens, F. nucleatum and S. mitis.* 34 % (20/58) of PA negative stable adult patients had anaerobes but they grew predominantly *Streptococcus anginosus, Streptococcus parasanguinis, P. melaninogenica* and *F. nucleatum*. Anaerobes in our study were present after treatment, and these anaerobes were often diverse.Worlitzsch and colleagues (Worlitzsch, Rintelen et al. 2009), found that intravenous therapy directed against *P. aeruginosa* during exacerbations increased lung function, but it did not however, reduce the

numbers of obligate anaerobes. Obligate anaerobes vary in their pattern of resistance and in their study. 58% of patients with acute exacerbations had obligate anaerobes that were resistant to the antibiotics used for treatment. Second generation sequencing has provided much more knowledge and insight into the complex diversity of the CF microbiome, however, the lack of clarity regarding the role of this newly discovered bacteria and their clinical implications needs to be elucidated.

One of the few studies published to show that anaerobes contributed to pathogenicity (Ulrich, Beer et al. 2010), found that *P. intermedia* was detected in high numbers $> 10^5$ CFU and that antibodies to the bacteria were recognised by > 90% of the patients with CF. Ulrich and colleagues, were also able to identify the proteases responsible and they found that *P. intermedia* grown anaerobically was more pathogenic than *P. aeruginosa* grown anaerobically. One of the most common anaerobes found in the CF lung, *P. melaninogenica,* (Tunney, Field et al. 2008), produces proteases. The main source of protease activity in the CF lung was thought to be activated neutrophils, however, bacterial proteases have been shown in previous studies mainly on *P. aeruginosa*, to activate cascade pathways, disrupt cytokine signalling and inactivate cell receptors (Quinn, Weldon et al. 2010). In our experiments, we have shown that proteases from *P. melaninogenica* can cleave antimicrobial peptides, lactoferrin and LL-37. These peptides are important in the first line of defence in the body. Interruption of this barrier, leads the host exposed to more fulminant disease. They also can inhibit the innate anti-proteases, AAT, SLPI and elafin from functioning. The release of

proteolytic enzymes during the inflammatory response allowing them to exacerbate and prolong inflammation, causes a number of deleterious effects, such as lung tissue destruction and reduce bacterial clearance. This protease and antiprotease balance is disrupted and the proteolytic destruction is increased, leading to further inhibition of naturally occurring anti-proteases such as AAT, SLPI and elafin. The high protease burden in the CF lung is turned on and potentially damaging degradation of host tissue is allowed to continue. This is a novel mechanism of attempting to explain the possible pathogenicity of one of the most common anaerobes in the CF lung.

Short chain fatty acids (SCFA) by activating GPR 41 and 43 can inhibit cAMP production and activation of the ERK cascade (Le Poul, Loison et al. 2003) .SCFAs can cause chemotaxis of neutrophils *in vitro* by activating GPR43 (Le Poul, Loison et al. 2003, Sina, Gavrilova et al. 2009, Vinolo, Ferguson et al. 2011).Using knockout mice, Malowski and colleagues have recently shown that acetate signalling through GPR 43 is a key regulator of inflammation. (Maslowski, Vieira et al. 2009). Furthermore, SCFA produced by bacterial vaginosis can induce IL-8, IL-6 and IL-1β release and this contributes to a pro-inflammatory milleu. SCFAs, by products from the fermentation of anaerobic bacteria were investigated for their pro-inflammatory effects in the CF lung. Key receptors for SCFA have been identified to exist in the CF lung and have shown in our experiments to have an increased expression in comparison with control cells. The SCFA on the CF cells had also increased pro-cytokine effects and caused an increase in IL-8 response. The cycle of inflammation continues, anaerobic

bacteria in the CF lung exist in high numbers, produce unique short chain fatty acids depending on the anaerobic bacteria produced and these SCFAs are mediated into the cell via receptors that are much more highly expressed in CF cells. Then, the SCFA on the cells can induce and increase IL-8 response and so the pro-inflammatory cycle continues (Figure 6.1).

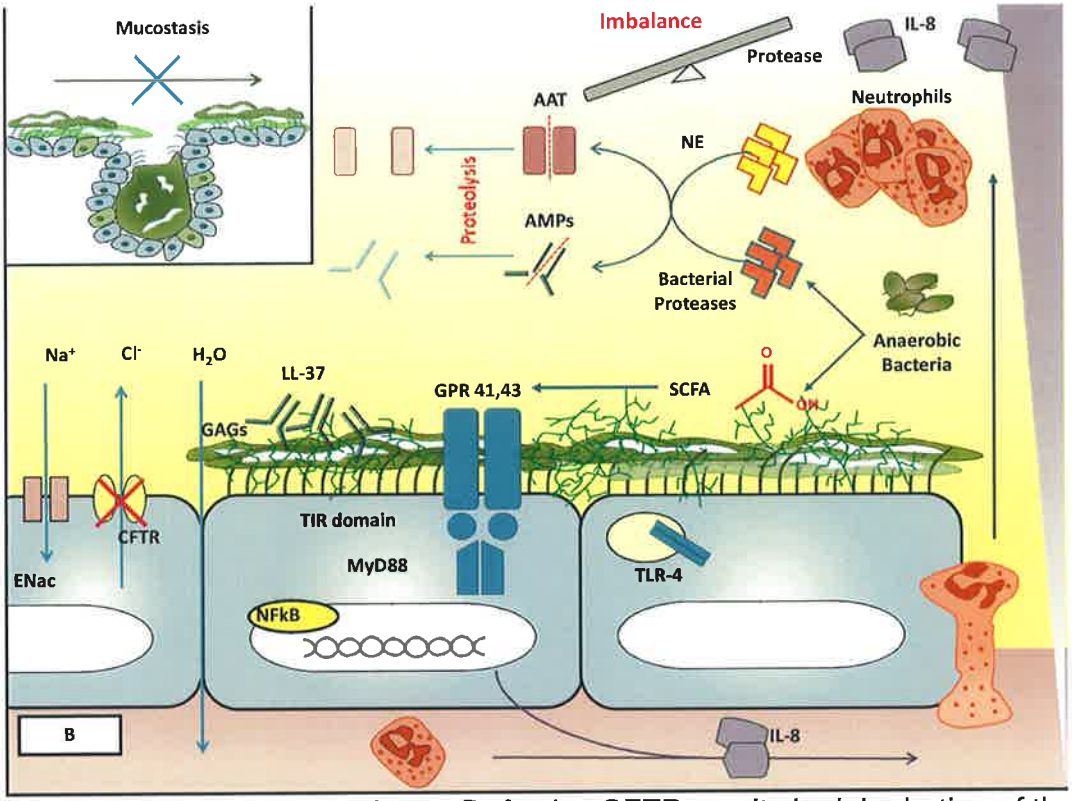


Figure 6.1: Cystic fibrosis airway: Defective CFTR results in dehydration of the ASL causing an impairment of the mucociliary escalator and a vicious circle of mucostasis, bacterial colonisation, infection and excessive neutrophilic inflammation. Disruption of the existing protease-antiprotease balance because of excessive amounts of bacterial and neutrophil derived proteases interferes with the innate immune response, including anti-microbial peptides (AMPs).) NE cleaves important anti-proteases and anti-microbial proteins in the CF lung including alpha-1 antitrypsin (AAT), secretory leukoprotease inhibitor (SLPI), and the cathelicidin: LL-37, elafin and lactoferrin. NE activates TLR (Toll-like receptor) signalling in conjunction with EGFR (Epidermal growth factor receptor) as well as PAR-2 (Protease activated receptor-2). This leads to up-regulation of pro-inflammatory cytokines such as interleukin 8 (IL-8). The major pathogen recognition receptors (PRRs) are the toll-like receptor (TLR) family upon contact with microbial derived factors such as flagellin from pseudomonas aeruginosa (PA). Short chain fatty acids produced as by products of anaerobic bacteria are mediated through the cell by G protein coupled receptors (GPR) 41 and 43. This also leads to upregulation of IL-8. ENaC: Epithelial sodium channel; CFTR: Cystic fibrosis transmembrane conductance regulator; IL-8: Interleukin-8; NE: Neutrophil elastase; PA: Pseudomonas aeruginosa; ML: Mucus layer; PCL: Periciliary layer.

6.2 Conclusion

Anaerobic bacteria existed in both paediatric and adult groups in high numbers, up to 58% in those colonised with *P.aeruginosa.* The diversity of anaerobic bacteria changed as the CF population aged and were colonised with known bacteria, *P. melaninogenica, F. nucleatum* and *Veillonella* species in increased numbers. Anaerobic bacteria did not grow on routine clinical cultures and as a result in clinical practice; there is need for better and improved molecular techniques that can easily be brought to clinical laboratories.

It is still remains largely unclear, the clinical implications of anaerobic bacteria. We have shown that proteases are produced by anaerobic bacteria and this has a dampening effect in vitro on the antimicrobial peptides and antiproteases. Thus, providing a mechanism for pathogenicity. Finally, SCFA produced by anaerobic bacteria, can be identified by the unique SCFA that they produce. The receptors for SCFA are over expressed on CF cells and SCFA can induce increased IL-8 response and be potentially responsible for continuous proinflammatory cycle.

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Chapter 7

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Appendix

Chapter 8

8.1 BH CONSENT FORM (including genetic and tissue retention)

Protocol Title:

The Role of Anaerobic Bacteria in Cystic Fibrosis

Please tick the appropriate answer.

I confirm that I have read and understood the Patient Information Leaflet dated _____ attached, and that I have had ample opportunity to ask questions all of which have been satisfactorily answered. Yes No

I am an adult taking part in this study. □ Yes □ No

I understand that my participation in this study is entirely voluntary and that I may withdraw at any time, without giving reason, and without this decision affecting my future treatment or medical care. Yes No

I understand that my records may be viewed by individuals with delegated authority from Professor NG McElvaney including members of the research team. I have been assured that information about me will be kept confidential. Yes No

I understand that my identity will remain confidential at all times. Yes No

I am aware of the potential risks of this research study. Yes No

I have been given a copy of the Patient Information Leaflet and this Consent form for my records. Yes No

I agree to donate samples (blood, sputum and urine) for this research project. I understand that given a sample for this research is voluntary and that I am free to withdraw my approval at any time without my medical treatment being affected. Yes No

Storage and Future uses of Biological Material

I give permission for my samples and information collected about me to be sorted for possible future research related to this study (including DNA or genetic studies) but only if obtained the research is approved by a Research Ethics Committee. Yes No

FUTURE USE OF ANONYMOUS DATA:

I agree that I will not restrict the use to which the results of this study may be put. I give my approval that unidentifiable data concerning my person may be stored or electronically processed for the purpose of scientific research and may be used in related or other studies in the future. (This would be subject to approval by an independent body, which safeguards the welfare and rights of people in biomedical research studies such as the Beaumont Hospital Ethics (Medical Research) Committee. Yes No

Patient _____ _____ Signature and dated Name in block capitals

To be completed by the Principal Investigator or his nominee.

I the undersigned, have taken the time to fully explained to the above patient the nature and purpose of this study in a manner that he/she could understand. I have explained the risks involved, the experimental nature of the treatment, as well as the possible benefits and have invited him/here to ask questions on any aspect of the study that concerned them.

Signature: Name in Block Capitals: Qualification: Date:

3 copies to be made: 1 for patient, 1 for PI and 1 for hospital records.

8.2 BH Patient Information Leaflet

Protocol Title:

The role of anaerobic bacteria in Cystic Fibrosis

Principal Investigator's Name: Noel Gerard McElvaney

Principal Investigator's Title: Professor of Medicine

Telephone No. of Principal Investigator: 01-8093764

You are being invited to take part in a clinical research study carried out at Beaumont Hospital. Before you decide whether or not you wish to take part, you should read the information provided below carefully and if you wish discuss it with your family or GP. You should clearly understand the risks and benefits of participating in this study so that you can make a decision that is right for you - this process is known as Informed Consent.

You are not obliged to take part in this study and failure to participate will have no effect on your current or future medical care.

You may change your mind at any time (before the start of the study or even after you have commenced the study) for whatever reason without having to justify your decision and without any negative impact on the care you will receive from the medical staff or hospital.

WHY IS THIS STUDY BEING DONE? You have been selected for potential inclusion because you attend our clinic for the management of your cystic fibrosis.As you may know, patients with cystic fibrosis suffer with infections of the lung which require antibiotic treatment either in hospital or at home. Some of these infections are caused by bacteria. We now know that some of these bacteria grow in the absence of oxygen (and these are called 'anaerobic bacteria) and that these bacteria can exist in the lungs of patients with cystic fibrosis. We do not yet know how important these bacteria are or whether they play a major role in the lung disease or function in cystic fibrosis.

The aim of this study is to see if these particular bacteria are present in significant numbers and if so whether they causing infections. We also want to determine if such bacteria require any specific antibiotic treatments.

We are asking you to give us a sputum sample - to detect these bacteria and at the same time provide a urine and blood sample to detect infection. There are 2 separate parts to this study - in the first part, you are asked to provide a single once-off set of blood, urine and sputum samples and in the second part to provide 3 further sets of samples (blood, urine, sputum) over 12-18 months. You can participate only in the first and not the second part of you wish.

If you develop a lung infection needing antibiotics after giving us your first set of samples, we will ask for your permission to obtain the further samples before and after your antibiotic treatment and again 4 weeks after finishing the antibiotics when you return to being well again.

If you cannot cough sputum easily for us if you decide to participate, we will offer you a nebuliser of hypertonic saline that will make you cough so that you can more easily produce sputum. This procedure will not take longer than 15-20 minutes and will be done together with your doctor and the physiotherapist. We will discuss this in more detail with you if this may be required in your case. After completing the nebuliser, we will collect the sputum sample needed for the study.

If as part of your routine clinical care you are having a bronchoscopy (camera test into the lungs) we will ask for your permission to use a small part of the samples taken for this research while most of the sample will go as normal to the lab for whatever tests your doctor has ordered.

WHO IS ORGANISING THIS STUDY?

Professor McElvaney and his research team are organising this study. It is our responsibility to conduct this study to the highest standard and provide any information and additionally answer any questions you may have about it. You can contact us at anytime with questions/concerns at the number provided.

HOW WILL IT BE CARRIED OUT?

The study will commence in July 2010 and last till June 2015 however you will only be involved either on a once off single occasion (part 1 of study) or up to a maximum 18 month period (where we take a few samples while you are well at regular intervals or if you develop an infection before and after antibiotics and when you are well again - part 2 of study). The study will take place in Beaumont Hospital under the management of Professor McElvaney and his research team and also in Cystic Fibrosis centres in North Carolina, USA and Northern Ireland, Belfast. We hope to recruit in total 40 cystic fibrosis patients from Beaumont hospital and you have been identified as a potential patient for inclusion.

Participants were selected by Professor McElvaney & his research team and approached over the phone or in clinic following which you have attended today to meet with us to be provided with more information about the study and its future potential benefits.

WHAT WILL HAPPEN TO ME IF I AGREE TO TAKE PART?

After today's meeting with a member of Professor McElvaney's research team, you will be given whatever time you require to make a decision regarding your participation in this study. You can then contact us with your decision and if you decide to participate, you will be invited back to sign a consent form and be given any further information/answer any questions you may have. On your visit back a sample of sputum, urine and blood will be taken. We will ask at that time if you would like to be involved in the 2nd part of the study where we aim to follow 25 (of the 40) patients to obtain 3 further sets of samples over a 12-18 month period.

BENEFITS:

Your participation will contribute to our overall results that could make a future difference to the treatments offered to patients with cystic fibrosis if we do find that these anaerobic bacteria are affecting the lungs in cystic fibrosis. It is important to note that you directly will not receive any results from this study.

RISKS:

There are minimal risks involved in the procedures being used in this study. These techniques as you will be aware are part of normal CF care, are relatively safe, non-invasive and provide us information we require to conduct our research.

Potential risks involve bruising, pain and discomfort associated with the use of needles during blood sampling and cough and throat irritation following sputum sampling.

Although the utmost precautions are always taken with your personal information, a potential breach of patient confidentiality is a risk with all studies and must be mentioned at this point.

WHAT IF SOMETHING GOES WRONG AS A RESULT OF MY PARTICIPATION IN THIS STUDY?

There are very few risks associated with your participation in our study. We do not anticipate any problems or adverse effects. You will however be provided a contact number at which you will be able to reach a member of the research team in the event of any worry, problem or question that may arise during your participation. If anything abnormal or unexpected is detected during your participation in the study, an appropriate medical referral will be made and medical care provided to address any such issues.

YOUR RESPONSIBILITIES AS A PARTICIPANT

If you agree to participate in this study, you will be asked to either provide a once-off sputum, blood and urine sample (Part 1 of study) and be invited to provide up to 3 further sample sets over 12-18 month period (Part 2 of study).

OUR RESPONSIBILITIES TO YOU AS INVESTIGATORS

If any new information becomes available to us during the course of the study that may in anyway affect your willingness to continue your participation, we will inform you immediately and it will be your decision whether or not to continue your participation in the study.

CONFIDENTIALITY ISSUES

Your GP will not be informed of your participation unless this is specifically requested by you. In this case, a member of Professor McElvaney's research team will contact your GP personally to let them know you are participating in this study.

All your information (including medical records) will be viewed only by members of Professor McElvaney's research team and coded to protect your identity. The information will be stored on encrypted, password protected computer systems. All your results and your personal information from the study will be stored for a period not exceeding ten years after which they will be destroyed as per hospital protocol.

All samples obtained in this study will be stored in appropriate conditions, coded to protect your identity and only be accessed by researchers authorised by Professor McElvaney. The samples will be kept for a period not exceeding ten years and be destroyed if not used further as per hospital protocol. It is important to note that as we are performing this study with our colleagues in the USA and Belfast that your sample may be sent to one or both of these laboratories however the samples will be 'coded' so that your identity is always kept confidential. Only Professor McElvaney or a member of his research team will be aware of the code and therefore your identity when looking at the data. This will not be shared with the USA or Belfast.

IF YOU REQUIRE FURTHER INFORMATION

If you have any further questions about the study, or if you wish to withdraw from the study you may do so without justifying your decision and your current and/or future treatment will not be affected in anyway.

For additional information now or any future time please contact:

Name: Dr Michelle Murray, Dr Kevin Molloy or Dr Zaza Abidin

Address: Department of Medicine, Respiratory Research Division, Education & Research Centre, Beaumont Hospital, Dublin 9

Phone No: (353) 01-8093801, 01-8093800

8.3 BH Control Information Leaflet

Protocol Title:

The Role of anaerobic bacteria in Cystic Fibrosis

Principal Investigator's Name: Noel Gerard McElvaney

Principal Investigator's Title: Professor of Medicine

Telephone No. of Principal Investigator: 01-8093764

You are being invited to take part in a clinical research study carried out at Beaumont Hospital. Before you decide whether or not you wish to take part, you should read the information provided below carefully and if you wish discuss it with your family or GP. You should clearly understand the risks and benefits of participating in this study so that you can make a decision that is right for you - this process is known as Informed Consent.

You are not obliged to take part in this study and failure to participate will have no effect on your current or future care.

You may change your mind at any time (before the start of the study or even after you have commenced the study) for whatever reason without having to justify your decision and without any negative impact on the care you will receive from the medical staff and hospital.

WHY IS THIS STUDY BEING DONE?

We are carrying out a study on the disease Cystic Fibrosis but although you don't have cystic fibrosis you have been selected for possible participation because we need to compare any samples we obtain from patients with Cystic Fibrosis to patients without Cystic Fibrosis (i.e. a patient like yourself). In research terms, this is called 'control' patients (i.e. patients who DO NOT have the condition which is being studied).

We will first introduce what this study is all about:

Cystic Fibrosis is Ireland's most common life-threatening inherited disease.

It damages many organs including the lungs, pancreas, digestive tract and the reproductive system. It causes thick sticky mucus to be produced, blocking the breathing tubes. A build up of this thick mucus can make it difficult to clear bacteria and leads to cycles of lung infections and inflammation, which can eventually lead to damage of the lungs and affect breathing.

Patients with Cystic Fibrosis suffer with infections which require antibiotic treatment either in hospital or at home. Some of these infections are caused by bacteria. We have discovered that some of these bacteria can grow when there is no oxygen available to them (these bacteria are called 'anaerobic' bacteria). We do not know at present how important these particular groups of bacteria are in Cystic Fibrosis. The aim of this study is to see if these bacteria are present in large numbers and if so are they causing frequent infections. We also want to check if these bacteria

require specific treatment. We also wish to see if these bacteria themselves are pathogenic and do they play a role in inflammation and if possible is it possible to reverse this.

We are asking you to be part of the study as we need to compare the cystic fibrosis patient samples to samples from patients who DO NOT have Cystic Fibrosis.

We are asking you to give us a sputum sample - to detect these bacteria and at the same time provide a urine and blood sample. If you cannot cough sputum up easily we can then give you a short nebuliser to make you cough and then we can collect the sputum after.

If part of your medical care includes having a bronchoscopy (camera test into the lungs) we will ask for your permission to use a small part of the samples taken for this research while most of the sample will go as normal to the lab for whatever tests your doctor has ordered.

If you are agreeable, we may ask you to provide over an 18 month period up to 3 further samples of sputum, urine and blood (every 3-6 months).

None of the procedures involved in obtaining sputum, urine or blood involve any major risk to yourself and will follow a routine procedure we use in clinics.

WHO IS ORGANISING THIS STUDY?

Professor McElvaney and his research team are organising this study. It is our responsibility to conduct this study to the highest standard and provide any information and additionally answer any questions you may have about it. You can contact us at anytime with questions/concerns at the number provided.

HOW WILL IT BE CARRIED OUT?

The study will commence in July 2010 and last till June 2015 however you will only be involved either on a once off single occasion or up to a maximum 18 month period. The study will take place in Beaumont Hospital under the management of Professor McElvaney and his research team and also in Cystic Fibrosis centres in North Carolina, USA and Belfast, Northern Ireland. We aim to recruit up to 40 patients from Beaumont hospital and you have been identified as a potential patient for inclusion.

Participants were selected by Professor McElvaney & his research team and approached over the phone or in clinic following which you have attended today to meet with us to be provided with more information about the study and its future potential benefits for Cystic Fibrosis patients.

WHAT WILL HAPPEN TO ME IF I AGREE TO TAKE PART?

After today's meeting with a member of Professor McElvaney's research team, you will be given whatever time you require to make a decision regarding your participation in this study. You can then contact us with your decision and if you decide to participate, you will be invited back to sign a consent form and be given any further information/answer any questions you have. On your visit back a sample of sputum and urine and blood will be taken. We will follow 25 of the 40 patients for up to 18 months and we will take sputum, urine and blood samples at regular intervals. You do not have to participate in this part if you wish.

BENEFITS:

Your participation will contribute to our overall results that could make a future difference to the treatments offered to patients with Cystic Fibrosis.

RISKS:

There are minimal risks involved in the procedures being used in this study. These are relatively safe, non-invasive and provide us information we require to conduct our research.

Potential risks involve bruising, pain and discomfort associated with the use of needles during blood sampling and minor cough and throat irritation following sputum sampling.

Although the utmost precautions are always taken with your personal information, a potential breach of patient confidentiality is a risk with all studies and must be mentioned at this point.

WHAT IF SOMETHING GOES WRONG AS A RESULT OF MY PARTICIPATION IN THIS STUDY?

There are very few risks associated with your participation in our study. We do not anticipate any problems or adverse effects. You will however be provided a contact number at which you will be able to reach a member of the research team in the event of any worry, problem or question that may arise during your participation. If anything abnormal or unexpected is detected during your participation in the study, an appropriate medical referral will be made and medical care provided to you to address any such issues.

YOUR RESPONSIBILITIES AS A PARTICIPANT

You are required to provide a sputum, blood and urine sample on one occasion and if you agree to participate for a longer period, up to three groups of samples over an 18 month period.

OUR RESPONSIBILITIES TO YOU AS INVESTIGATORS

If any new information becomes available to us during the course of the study that in anyway may affect your willingness to continue your participation, we will inform you immediately and it will be your decision whether or not to continue your participation.

CONFIDENTIALITY ISSUES

Your GP will not be informed of your participation unless this is specifically requested by you. If you request us to inform your GP, a member of Professor McElvaney's research team will contact him/her personally.

All your information (including medical records) will be viewed only by members of Professor McElvaney's research team and be stored on encrypted, password protected computer systems. All your results and your personal information from the study will be stored for a period not exceeding ten years after which they will be destroyed as per hospital protocol. All your information within this system will be 'coded' (which means that nobody except someone who can break the code can identify who you are). The only people able to break this code are Professor McElvaney and a select few members of his research team. Your personal information (in a coded fashion so that you cannot be identified) may be shared with our other research partners in the USA (North Carolina) and Northern Ireland (Belfast).

All samples obtained in this study will be stored and again coded to protect your identity. These samples can only be accessed by researchers authorised by Professor McElvaney. The samples will be kept for a period not exceeding ten years and be destroyed if not used further as per hospital protocol. As this study will be carried out in Dublin, Belfast and USA, some of the sample processing may be done outside of Professor McElvaney's lab in Dublin. In such cases, your identity will be confidential and only the sample sent to our partner lab. All data (including samples) we collect through this research study will be 'coded'. This means that only members of Professor McElvaney's research team will be aware of the code and therefore your identity when looking at the data not even our partners in Belfast or the USA.

IF YOU REQUIRE FURTHER INFORMATION

If you have any further questions about the study, or if you wish to withdraw from the study you may do so without justifying your decision and your future treatment will not be affected.

For additional information now or any future time please contact:

Name: Dr Michelle Murray, Dr Kevin Molloy or Dr Zaza Abidin

Address: Department of Medicine, Respiratory Research Division, Education & Research Centre, Beaumont Hospital, Dublin 9

Phone No: (353) 01-8093801, 01-8093800

8.4 Assent Forrm (Children 6-12 years old)

I have read the leaflet the doctors gave to me about this study.

I understand what the study is about

I am happy to give some of my sputum for this study

I know that if I don't want to take part in this study I can change my mind whenever I want.

I know that I will only need to give some of my sputum if I have any.

I have been given a chance by the doctor to ask any questions that I have about this study and I am happy with what they said.

I am happy for my parents/guardian to sign the form to consent for me to take part in this study.

Name Date of Birth

Signed Date

8.5 Assent Form (Children 12-18 years old)

I confirm that I have read and understand the information leaflet given to me about this study.

I agree to donate sputum samples for this study.

I understand that I will only need to give a sputum sample if I have any.

I was given plenty of time to consider whether to take part in this study and discussed this with my parents/guardian.

I understand that my participation in this study is voluntary and I can withdraw at any time.

I have been given a chance by my doctor to ask any questions that I have about this study and I am happy with their answers.

I allow my guardian to consent on my behalf to take part in this study.

Name Date of Birth

Signed Date

8.6 Consent Form

Title of Study: The Role of Anaerobic Bacteria in Cystic Fibrosisesearch Participant's Name:

Name of Doctor and Telephone Number: Dr Paul McNally (01 4096 100

I confirm that I have read and understand the information leaflet dated ..... for the above study and received an explanation of the nature, purpose, duration, and foreseeable effects and risks of the study and what my/my child's involvement will be.

I have had time to consider on my child's behalf, whether to take part in this study. My questions have been answered satisfactorily and I have received a copy of the Patient Information Leaflet.

I understand that my child's participation is voluntary and that we are free to withdraw at any time without affecting my child's medical care or legal rights.

I have to the best of my knowledge informed the investigator of my child's previous or present illness and medication and of any consultation that my child had with a doctor for the last four months. My child has not participated in any other clinical trial in the past four months.

I understand that my General Practitioner will be informed (if requested) that my child is taking part in the study

I am willing to allow access to my child's medical records by the researchers, Ethics Committee or local or foreign regulatory authorities but understand that strict confidentiality will be maintained. The purpose of this is to check that the study is being carried out correctly.

I agree to donate samples (bronchoalveolar lavage fluid or sputum) from my child for this research project. I understand that giving a sample for this research is voluntary and that I am free to withdraw my approval at any time without my child's medical treatment being affected.

I give permission for the samples and information collected about my child to be stored for possible future research related to this study but only if the research is approved by a Research Ethics Committee.

I agree to allow my child to take part in the above study.

Name of Research Participant/Parent/Guardian Date Signature (in block letters)

Name of Person taking consent Date Signature (if different from doctor/researcher)
Doctor/Researcher Date Signature

1 copy for research participant 1 copy for researcher 1 copy to be inserted into the hospital notes

8.7 Control Information Leaflet

Protocol Title:

The Role of anaerobic bacteria in Cystic Fibrosis

Principal Investigator's Name: Dr Paul McNally

Principal Investigator's Title: Consultant Respiratory Physician

Telephone No. of Principal Investigator: 01-4096100

Your child is being invited to take part in a clinical research study carried out at Our Lady's Children's Hospital Crumlin. Before you decide whether or not you wish to take part, you should read the information provided below carefully and if you wish discuss it with your family or GP. You should clearly understand the risks and benefits of participating in this study so that you can make a decision that is right for your child - this process is known as Informed Consent.

Your child is not obliged to take part in this study and failure to participate will have no effect on your child's current or future medical care.

You may change your mind at any time (before the start of the study or even after you have commenced the study) for whatever reason without having to justify your decision and without any negative impact on the care your child will receive from the medical staff or hospital.

WHY IS THIS STUDY BEING DONE?

We are currently conducting a study looking at lung infection in children with cystic fibrosis. As part of this study, will be using some complicated laboratory techniques to try and find out more about infection in the lungs of children with cystic fibrosis. The results that these tests give us can be difficult to interpret -- Are they due to cystic fibrosis? Would they be the same in all children? The best way to work this out is to compare samples from children with cystic fibrosis those from children who DO NOT have cystic fibrosis.

We will first introduce what this study is all about:

Cystic Fibrosis is Ireland's most common life-threatening inherited disease. It damages many organs including the lungs, pancreas, digestive tract and the reproductive system. It causes thick sticky mucus to be produced, blocking the breathing tubes. A build up of this thick mucus can make it difficult to clear bacteria and leads to cycles of lung infections and inflammation, which can eventually lead to damage of the lungs and affect breathing.

Patients with Cystic Fibrosis suffer with infections which require antibiotic treatment either in hospital or at home. Most of these infections are caused by bacteria. We have discovered that some of these bacteria can grow when there is no oxygen available to them (these bacteria are called 'anaerobic' bacteria). We do not know at present how important these particular groups of bacteria are in Cystic Fibrosis. The aim of this study is to see if these bacteria are present in large numbers and if so are they causing frequent infections. We also want to check if these bacteria require specific treatment.

We are asking your child to be part of the study as we would like to compare the samples from children with Cystic Fibrosis to samples from patients who DO NOT have Cystic Fibrosis.

Your child is being asked to participate in this study because they are undergoing a camera test to look at their windpipe. This test is called flexible bronchoscopy. This procedure is done by your doctor in order to determine the cause of the airway problem that your child is experiencing. This test is done under the general anaesthesia and your child will not feel any pain. Washing of the windpipe is routinely done during this procedure; this is called bronchoalveolar lavage fluid (BAL). The fluid from this washing is usually sent to the laboratory for processing.

When the laboratory analyses the BAL sample they only use a small portion of it; the remainder is discarded. We consider this a waste of a precious sample which could help answer many important questions about infection and inflammation in the lungs of young children. We plan to introduce a study which will keep the extra sample, rather than destroy it, to allow detailed investigations of the bacterial that causes infections and inflammation in the lungs. Your child will be invited to participate in that study when it begins. To prepare for that study we plan to use the fluid that is leftover from the BAL samples.

You will sign a separate consent form to allow the flexible bronchoscopy and BAL to proceed. This information sheet and the attached consent form, is to allow us to keep the leftover BAL sample and perform further laboratory analysis on it, and to perform the bronchial brushing, and to analyse the sample obtained.

WHO IS ORGANISING THIS STUDY?

Dr Paul McNally and Dr Gerard Canny, The Respiratory Consultants in this hospital (OLCHC) are working with Professor Gerard McElvaney, a CF Consultant in Beaumont and their teams are organising this study. It is our responsibility to conduct this study to the highest standard and provide any information and additionally answer any questions you may have about it. You can contact us at anytime with questions/concerns at the number provided.

HOW WILL IT BE CARRIED OUT?

The study will commence in September 2010 and last until June 2015. We will take a once-off BAL sample from your child. Only excess samples will be used for this study. These samples are usually discarded and there will be no extra procedure that will be done purely for this research.

The processing of samples for this study will take place in Beaumont Hospital under the management of Professor McElvaney and his research team and also in the Cystic Fibrosis centres in North Carolina, USA and Belfast. We hope to recruit in total 25 children without the diagnosis of CF from Our Lady's Children's Hospital, Crumlin and your child have been identified as a potential patient for inclusion.

The design of the paediatric component of this study was undertaken by Dr Paul McNally, one of the CF Consultants in this hospital (OLCHC) and Professor Gerard McElvaney, CF Consultant in Beaumont Hospital. Both Dr McNally and Dr Canny are aware of the details of the study and are happy for it to proceed in Crumlin. All Consultants and the research team are happy to provide you with more information about the study and its future potential benefits.

WHAT WILL HAPPEN TO ME / MY CHILD IF I AGREE TO TAKE PART?

After today's meeting with a member of the research team, you will be given whatever time you require to make a decision regarding your child's participation in this study. You can then contact us with your decision and if you decide to participate, you will be invited back to sign a consent form and be given any further information/answer any questions you may have.

BENEFITS:

Your participation will contribute to our overall results that could make a future difference to the treatments offered to patients with cystic fibrosis if we do find that these anaerobic bacteria are affecting the lungs in cystic fibrosis. It is important to note that you directly will not receive any results from this study.

RISKS:

There are minimal risks involved in the procedures being used in this study. Almost all medical investigations and treatments have some risks. For example; one of the risks of having a blood test is that it may cause pain and distress. Most of the procedures we perform on a daily basis have only minor risks associated with them, and the benefit to the patient is more important than the risk associated with it.

Your child does not have to undergo any extra procedure purely for this research purposes.

The risks associated with the performance of routine flexible bronchoscopy and bronchoalveolar lavage will be explained to you as part of obtaining your informed consent for the procedure, which you will have signed. No pain or discomfort is felt during bronchoscopy because your child is under anaesthetic.

EXCLUSION FROM PARTICIPATION:

Children with insufficient sample will be excluded from this study. If your child is scheduled for bronchoscopy and is unwell at the time, they will not be included for this study. However this is unlikely to occur, as children who are unwell will usually have their bronchoscopy deferred and rescheduled.

ALTERNATIVE MANAGEMENT:

You do not have to be part of this study to receive the highest quality care available from this unit. All children will receive the optimum care available.

VOLUNTARY PARTICIPATION:

You have consented for your child to participate in this study. You may quit at any time. If you decide not to participate, or if you quit, you will not be penalised and will not give up any benefits which you had before entering the study. You are free at any time to withdraw from the study. If you withdraw from the study you will have the opportunity to have all research samples collected from your child destroyed.

CONFIDENTIALITY ISSUES

Your GP will not be informed of your child's participation unless this is specifically requested by you. In this case, a member of the research team will contact your GP personally to let them know your child is participating in this study.

All the information (including medical records) from your child will be viewed only by members of the research team and coded to protect your child's identity. The information will be stored on encrypted, password protected computer systems. All the results and personal information from the study will be stored for a period not exceeding ten years after which they will be destroyed as per hospital protocol.

All samples obtained in this study will be stored in appropriate conditions, coded to protect your child's identity and only be accessed by researchers authorised by Dr McNally, Dr Canny and Professor McElvaney. The samples will be kept for a period not exceeding ten years and be destroyed if not used further as per hospital protocol. It is important to note that as we are performing this study with our colleagues in the USA and Belfast and that the sample may be sent to one or both of these laboratories however the samples will be 'coded' so that the identity is always kept confidential. All of the clinical details of your child will be held in OLCHC at all times.

IF YOU REQUIRE FURTHER INFORMATION

If you have any further questions about the study or if you wish to withdraw from the study you may do so without justifying your decision and your child's current and/or future treatment will not be affected in anyway.

For additional information now or any future time please contact:

Name: Dr Michelle Murray, Dr Kevin Molloy or Dr Zaza Abidin

Address: Department of Medicine, Respiratory Research Division, Education & Research Centre, Beaumont Hospital, Dublin 9

Phone No: (353) 01-8093801, 01-8093800

8.8 Parent Information Leaflet

The role of anaerobic bacteria in Cystic Fibrosis

Principal Investigator's Name: Dr Paul McNally

Principal Investigator's Title: Consultant Respiratory Physician

Telephone No. of Principal Investigator: 01-4096100

Your child is being invited to take part in a clinical research study carried out at Our Lady's Children's Hospital Crumlin. Before you decide whether or not you wish to take part, you should read the information provided below carefully and if you wish discuss it with your family or GP. You should clearly understand the risks and benefits of participating in this study so that you can make a decision that is right for your child - this process is known as Informed Consent.

Your child is not obliged to take part in this study and failure to participate will have no effect on your child's current or future medical care. You may change your mind at any time (before the start of the study or even after you have commenced the study) for whatever reason without having to justify your decision and without any negative impact on the care your child will receive from the medical staff or hospital.

WHY IS THIS STUDY BEING DONE?

Your child has been considered for inclusion in the study because he/she attends our clinic for the management of cystic fibrosis.

As you know, children with cystic fibrosis often get lung infections which require antibiotic treatment either in hospital or at home. Most of these infections are caused by bacteria. We now know that some of these bacteria grow in the absence of oxygen (and these are called 'anaerobic' bacteria) and that these bacteria can exist in the lungs of children with cystic fibrosis. We do not yet know how important these bacteria are or whether they play a major role in CF lung disease or affect lung function in cystic fibrosis.

The aim of this study is to see if these particular bacteria are present in significant numbers and if so whether they are causing problems. We also want to determine if such bacteria require any specific antibiotic treatments.

If your child is between 0 - 6 years old, we will collect the fluid from airway washings (bronchoscopy procedure). As infants and young children do not cough up phlegm it is now established that bronchoalveolar lavage (BAL) is the best test to detect infection in the lungs. The BAL sample is obtained by performing a flexible bronchoscopy under general anaesthetic. This is performed every year on all babies and young children with CF attending Our Lady's Children's Hospital, Crumlin (OLCHC) up to six years of age. When the laboratory analyses the BAL sample they only use a small portion of it; the remainder is discarded. We consider this a waste of a precious sample which could help answer many important questions about infection and inflammation in the lungs of young children with CF. We plan to introduce a study which will keep the extra sample, rather than destroy it, to allow detailed investigations of the bacterial that causes infections in the CF lung. Your child will be invited to participate in that study when it begins. To prepare for that study we plan to use the fluid that is leftover from the BAL samples.

If your child is between 6 - 18 years old, we will be collecting sputum samples. This will be done when your child attends the clinic. Routinely your child will be given a sputum pot in the clinic on each visit. The sample is usually sent to the lab for analysis. For this research purposes, we are not asking your child to produce any extra sample. We will only use excess sample and your child will not have to undergo any extra procedure to provide their sputum sample.

There are 2 separate parts to this study for children aged 6 - 18 years old. In the first part, your child is asked to provide a single once-off sample and in the second part to provide 3 further samples over 12-18 months. Your child can participate only in the first and not the second part of the study if they wish.

If your child develops a lung infection needing antibiotics after giving us the first sample, we will ask for your permission to obtain the further samples before and after your child's antibiotic treatment and again 4 weeks after finishing the antibiotics when he / she returns to being well again. We will only take excess sample from your child. We will not subject your child to provide us with extra sputum and they will not have to undergo any extra procedure.

WHO IS ORGANISING THIS STUDY?

Dr Paul McNally and Dr Gerrard Canny, The Respiratory Consultants in this hospital (OLCHC) are working with Professor Gerard McElvaney, a CF Consultant in Beaumont and their teams are organising this study. It is our responsibility to conduct this study to the highest standard and provide any information and additionally answer any questions you may have about it. You can contact us at anytime with questions/concerns at the number provided.

HOW WILL IT BE CARRIED OUT?

The study will commence in September 2010 and last until June 2015. There are 2 parts in this study (Part 1 and Part 2). Part 1 of this study involves a once-off collection of samples. For children aged between 0 - 6 years samples will be taken from bronchoscopy. For children aged 6 - 18 years we ask for a sputum sample during routine clinic visit. Only excess samples will be used for this study. These samples are usually discarded and there will be no extra procedure that will be done purely for this research.

Part 2 of this study involves following up children aged 6 - 18 years. Your child will be followed up for a period of 12 - 18 months. We will see them during their routine clinic follow up (every 3 months) and sputum sample will be collected on each visit. If during this interim period your child develops infection and requires antibiotic treatment, we will ask for sputum prior to starting antibiotic treatment, after completing treatment and when your child is well again (3 samples in total). This will be done in the hospital during admission and clinic visit, and is part of their routine clinical care. Your child is not required to produce any extra sample for this study. We will only use excess sample for this research.

The processing of samples for this study will take place in Beaumont Hospital under the management of Professor McElvaney and his research team and also in the Cystic Fibrosis centres in North Carolina, USA and Belfast. We hope to recruit in total 50 cystic fibrosis patients from Our Lady's Children's Hospital, Crumlin and your child have been identified as a potential patient for inclusion.

The design of the paediatric component of this study was undertaken by Dr Paul McNally, one of the CF Consultants in this hospital (OLCHC) and Professor Gerard McElvaney, CF Consultant in

Beaumont Hospital. Both Dr McNally and Dr Canny are aware of the details of the study and are happy for it to proceed in Crumlin. All Consultants and the research team are happy to provide you with more information about the study and its future potential benefits.

WHAT WILL HAPPEN TO ME / MY CHILD IF I AGREE TO TAKE PART?

After today's meeting with a member of the research team, you will be given whatever time you require to make a decision regarding your child's participation in this study. You can then contact us with your decision and if you decide to participate, you will be invited back to sign a consent form and be given any further information/answer any questions you may have. On your visit back a sample of sputum or in the case of a bronchoscopy a fluid sample will be taken. We will ask at that time if you would like to be involved in the 2nd part of the study where we aim to follow up a group of patients to obtain 3 further sets of samples over a 12-18 month period as described earlier.

BENEFITS:

Your participation will contribute to our overall results that could make a future difference to the treatments offered to patients with cystic fibrosis if we do find that these anaerobic bacteria are affecting the lungs in cystic fibrosis. It is important to note that you directly will not receive any results from this study.

RISKS:

There are minimal risks involved in the procedures being used in this study. These techniques as you will be aware are part of normal CF care, are relatively safe and provide us information we require to conduct our research. Almost all medical investigations and treatments have some risks. For example; one of the risks of having a blood test is that it may cause pain and distress. Most of the procedures we perform on a daily basis have only minor risks associated with them, and the benefit to the patient is more important than the risk associated with it.

The risks associated with the performance of routine flexible bronchoscopy and bronchoalveolar lavage will be explained to you as part of obtaining your informed consent for the procedure, which you will have signed. No pain or discomfort is felt during bronchoscopy because your child is under anaesthetic.

EXCLUSION FROM PARTICIPATION:

Children with insufficient sample will be excluded from this study. If your child is scheduled for bronchoscopy and is unwell at the time, they will not be included for this study. However this is unlikely to occur, as children who are unwell will usually have their bronchoscopy deferred and rescheduled.

ALTERNATIVE MANAGEMENT:

You do not have to be part of this study to receive the highest quality care available from this unit. All children will receive the optimum care available.

VOLUNTARY PARTICIPATION:

You have consented for your child to participate in this study. You may quit at any time. If you decide not to participate, or if you quit, you will not be penalised and will not give up any benefits

which you had before entering the study. You are free at any time to withdraw from the study. If you withdraw from the study you will have the opportunity to have all research samples collected from your child destroyed.

CONFIDENTIALITY ISSUES

Your GP will not be informed of your child's participation unless this is specifically requested by you. In this case, a member of the research team will contact your GP personally to let them know your child is participating in this study.

All the information (including medical records) from your child will be viewed only by members of the research team and coded to protect your child's identity. The information will be stored on encrypted, password protected computer systems. All the results and personal information from the study will be stored for a period not exceeding ten years after which they will be destroyed as per hospital protocol.

All samples obtained in this study will be stored in appropriate conditions, coded to protect your child's identity and only be accessed by researchers authorised by Dr McNally, Dr Canny and Professor McElvaney. The samples will be kept for a period not exceeding ten years and be destroyed if not used further as per hospital protocol. It is important to note that as we are performing this study with our colleagues in the USA and Belfast and that the sample may be sent to one or both of these laboratories however the samples will be 'coded' so that the identity is always kept confidential. All of the clinical details of your child will be held in OLCHC at all times.

IF YOU REQUIRE FURTHER INFORMATION

If you have any further questions about the study or if you wish to withdraw from the study you may do so without justifying your decision and your child's current and/or future treatment will not be affected in anyway.

For additional information now or any future time please contact:

Name: Dr Michelle Murray, Dr Kevin Molloy or Dr Zaza Abidin

Address: Department of Medicine, Respiratory Research Division, Education & Research Centre, Beaumont Hospital, Dublin 9

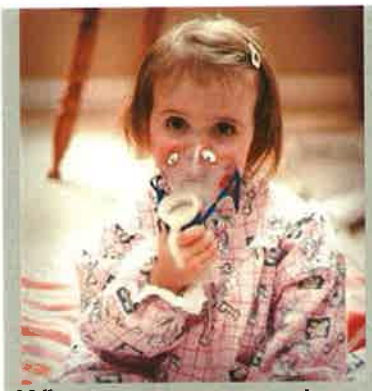
Phone No: (353) 01-8093801, 01-8093800

8.9 Patient Information Leaflet

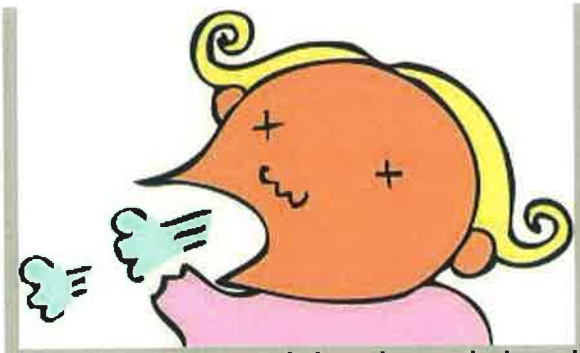
Hello there! We are the lung doctors working with Dr Paul McNally. We are from Beaumont Hospital.

We are here to get your permission to take some of your mucus spit from cough.

We will go through with you what we will do.

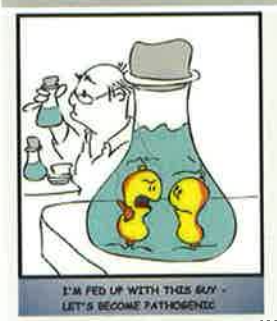


When you come to see Dr McNally we will check how you are doing. If you are coughing up a lot of mucus we would like to ask if you don't mind giving us some.



We will put your spit in a jar and close it. It is okay if you don't have any.

We will bring the spit to our LAB in Beaumont Hospital to have a



In the LAB, we will have a closer look at your spit and see the bugs that are in your spit

closer look.

After we have identified the bad bugs in your spit we will find the best antibiotic to kill them and stop you from becoming sick.

If you are healthy and well in one year we will see you each time you come to our clinic and take your spit if you have any.

causing you to become sick.

If you like us we can still see you after the first time. We will take more spit from you to bring in the LAB over one year. If you become unwell and need to see Dr McNally, we will collect your mucus again.

We are looking at mucus from children here, Belfast and America. When we finish with this experiment we can find the best antibiotic to treat the bugs that causing you and other children with CF to become sick and help you and other children to become well again.

If you have any questions we are here and happy to talk to you.

You can discuss with your parents before giving us your mucus spit.

If you don't feel like giving us your mucus spit it is absolutely okay. Nothing will change and Dr McNally will still see you in the clinic.

Dr Zaza Abidin

Dr Michelle Murray

Dr Kevin Molloy

8.10 Patient Information Leaflet

Protocol Title:

The role of anaerobic bacteria in Cystic Fibrosis

Principal Investigator's Name: Dr Paul McNally

Principal Investigaor's Title: Consultant Respiratory Physician

Telephone No. of Principal Investigator: 01-4096100

You have been invited to take part in a clinical research study carried out at Our Lady's Children's Hospital Crumlin. Before you decide whether or not you wish to take part, you should read the information provided below carefully and if you wish discuss it with your family. You should clearly understand the risks and benefits of participating in this study so that you together with your parents can make a decision that is right for you.

You do not have to take part in this study and choosing not to take part will not affect the care you receive at the hospital or in the clinic.

You may change your mind at any time (before the start of the study or even after you have commenced the study) for whatever reason without having to explain why you have changed your mind.

WHY IS THIS STUDY BEING DONE?

You have been considered for the study because you are attending our clinic for the management of cystic fibrosis.

As you know people who have cystic fibrosis sometimes get lung infections and need treatment with antibiotics. Some of the bacteria that cause the lung infections can grow in the lungs without the presence of oxygen. We call them 'anaerobic bacteria'. The reason why we are conducting this research is to find out how important these anaerobic bacteria infections are in CF patients and whether they worsen lung function.

We also want to know if such bacteria require any specific antibiotic treatments that we may be able to change in the future to better treat these infections.

We will ask to collect sputum samples from you. This will be done when you attend the clinic using the sample you usually give us. We will send what is needed to the lab as usual and then use the rest of your sample for this research.

There are 2 separate parts to this study. In the first part, we will request a single once-off sample from you and in the second part to provide 3 further samples over 12-18 months. You can participate only in the first and not the second part of the study if you wish and whatever you decide will in no way affect your treatments. The 3 extra samples over 12-18 months will be

collected in exactly the same way as the 1st sample and we will use the extra available from the sample you give in clinic.

If you develop a lung infection needing antibiotics after giving us the first sample, we will ask for your permission to obtain the further samples before and after your antibiotic treatment and again 4-6 weeks after finishing the antibiotics when you are well again. Again, we will send what is needed to the lab as usual and then use the rest of your sample for the research project. We will not ask you to produce more sputum that you feel able to, and you will not have to undergo any extra procedures.

WHO IS ORGANISING THIS STUDY?

The Respiratory Consultants in this hospital (OLCHC) Dr McNally and Dr Canny are working with Professor McElvaney, a CF Consultant in Beaumont hospital with their teams to organise and run this study. It is our responsibility to conduct this study to the highest standard and provide any information you need and additionally answer any questions you or your parents may have about it. You or your parent may contact us at anytime with questions/concerns at the number provided.

HOW WILL IT BE CARRIED OUT?

The study will commence in September 2010 and last until June 2015. There are 2 parts in this study (Part 1 and Part 2) as described above. You may participate in the first and not the second part if you wish. We will ask to use any extra sputum from the sample that you provide during clinic as described above.

The processing of samples for this study will take place in Beaumont Hospital under the management of Professor McElvaney and his research team and also in Cystic Fibrosis centres in North Carolina, USA and Belfast, Northern Ireland. We hope to recruit in total 50 cystic fibrosis patients from Our Lady's Children's Hospital, Crumlin and you have been identified as a potential patient for inclusion.

The design of the paediatric component of this study was undertaken by Dr McNally and Dr Canny, the CF Consultants in this hospital (OLCHC) and Professor McElvaney, CF Consultant in Beaumont Hospital. Both Dr McNally and Dr Canny are aware of the details of the study and are happy for it to proceed in Crumlin. All Consultants and the research team are happy to provide you and your parents with more information about the study and its future potential benefits if you wish.

WHAT WILL HAPPEN TO ME IF I AGREE TO TAKE PART?

After today's meeting with a member of the research team, you will be given whatever time you require to make a decision regarding your participation in this study. You or your parent can then contact us with your decision and if you decide to participate, you and your parent will be invited back to sign a consent form and be given any further information/answer any questions you or your parent may have. On your visit back a sample of sputum will be taken. We will ask at that time if you would like to be involved in the 2nd part of the study where we aim to follow you up to obtain 3 further samples over a 12-18 month time frame as described above.

BENEFITS:

Your participation will contribute to our overall results that could make a future difference to the treatments offered to patients with cystic fibrosis. It is important to note that you will not receive

any results directly from the samples that are taken for this study. Samples taken at the same time as the study sample will be sent to the lab and you will receive the results for these.

RISKS:

There are not direct risks involved in obtaining sputum samples from you.

EXCLUSION FROM PARTICIPATION:

If you have insufficient extra sample that we can use you will be excluded from this study.

ALTERNATIVE MANAGEMENT:

You do not have to be part of this study to receive the highest quality care available from this unit. All patients will receive the optimum care available.

VOLUNTARY PARTICIPATION:

Your parent has to sign the forms to agree for you to participate in this study only when you have agreed to. Remember you may quit at any time. If you decide not to participate, or if you quit, you will not be penalised and will not give up any benefits which you had before entering the study. You are free at any time to withdraw from the study. If you withdraw from the study your sputum samples will be discarded.

CONFIDENTIALITY ISSUES

Your GP will not be informed of your participation unless this is specifically requested by you or your parent. In this case, a member of the research team will contact your GP personally to let them know you are participating in this study.

All samples obtained in this study will be stored in appropriate conditions, coded to protect your identity and only be accessed by researchers authorised by Dr McNally, Dr Canny and Professor McElvaney. The samples will be kept for a period not exceeding ten years and be destroyed if not used further. It is important to note that as we are performing this study with our colleagues in the USA and Belfast and that the sample may be sent to one or both of these laboratories however the samples will be 'coded' so that the identity is always kept confidential. All of your clinical details will be held in OLCHC at all times.

IF YOU REQUIRE FURTHER INFORMATION If you or your parent have any further questions about the study or if you wish to withdraw from the study you may do so without justifying your decision and your current and/or future treatment will not be affected in anyway.

For additional information now or any future time please contact:

Name: Dr Michelle Murray, Dr Zaza Abidin or Dr Kevin Molloy

Address: Department of Medicine, Respiratory Research Division, Education & Research Centre, Beaumont Hospital, Dublin 9

Phone No: (353) 01-8093801, 01-8093800

8.11 Anaerobic Database

Anaerobic Organisms in Cystic Fibrosis Lung Disease

Subject ID# ____ Subject Initials ____ DOB __/__/__

Subgroup: _____(related to SpecAim, i.e. Cross-Sect., Longitudinal St or E)_

Gender: ☐ Male ☐ Female

CF ☐ No ☐ Yes

Year of CF Diagnosis: ____

Genotype: _____/_____

Sweat Cl (mMol/L) : _____

Ethnicity: ☐ Caucasian ☐ Hispanic ☐ African American ☐ Other (specify)

CF Manifestations/Complications

Pancreatic Status: ☐ PI ☐ PS

Sinusitis/polyps requiring surgery ☐ No ☐ Yes Year of Surgery ____

Medical History: In the past 30 days has the patient had any of the following?

Oral Antibiotics:	☐ No ☐ Yes	☐ currently taking ☐ stopped: date __
IV antibiotics:	☐ No ☐ Yes	start date ____ end date ____
Chronic Fluclouxacillin	☐ No ☐ Yes	
Chronic Azithromycin	☐ No ☐ Yes	
Inhaled antibiotics:	☐ No ☐ Yes	☐ Tobramycin ☐ Colistin
Oral Corticosteroids:	☐ No ☐ Yes	☐ currently taking ☐ stopped: date __
Inhaled Corticosteroids:	☐ No ☐ Yes	

Dental History:

Self reported: Caries ☐ No ☐ Yes Gum problems, bleeding ☐ No ☐ Yes

Physician Diagnosed Periodontitis ☐ No ☐ Yes

Other studies involved _____(observational studies only)

_____(observational

Lab Results

PFT Date ___/___/____ FEV1 _____ (L) FEV1 predicted _____% FEF25-75 _____ (L) FEF25-75 predicted _____%

Below CFF mean for age No ☐ Yes ☐

BALF Collected No ☐ Yes ☐ BALF: Total ml _____ Number of aliquots: _____

Date ___/___/____
Indication_____

Approach _____ In/Out _____/_____ % Return: _____

Cell Counts Total _____x 10^6 Mac _____% PMN _____% EPI _____% LY _____% EOS _____%

Micro

AFB: + / - *Virus:* + / - Which: _____

Aerobic Culture = clinical lab

Bacteria 1 _____ Quantity (*k) _____

Bacteria 2 _____ Quantity (*k) _____

Bacteria 3 Quantity (*k)

Sputum Collected (Clinical lab results) No ☐ Yes ☐ Date ___/___/____

P aer Smooth No ☐ Yes ☐ Quantity (1-4+) _____

P aer Mucoid No ☐ Yes ☐ Quantity (1-4+) _____

Serum collected No ☐ Yes ☐ Date ___/___/____ Serum Total ml _____ Number of aliquots:

Chest X-ray Date ___/___/____ Score: _____

Chest CT Date ___/___/____

WCC on admission for exacerbation Post exacerbation Done No ☐ Yes ☐

WBC_____x10^{9th} / liter WBC_____x10^{9th} / liter

Comments: